European Food Safety Authority

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## SCIENTIFIC OPINION

# Scientific Opinion on the safety of advantame for the proposed uses as a food additive[1]

**EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)**[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

## ABSTRACT

The Panel on Food Additives and Nutrient Sources added to Food (ANS) provides a scientific opinion on the safety of advantame as a sweetener for use in the food categories specified in the dossier. Advantame is stable under normal storage conditions. The Panel noted that there is an indication of advantame instability in acidic beverages and thermally treated foods. Metabolism and toxicokinetics of advantame and its main metabolite, ANS9801-acid, have been studied in mice, rats, rabbits, dogs and humans. Advantame is rapidly but poorly absorbed and the main excretion route is via faeces. The Panel concluded that advantame does not raised concern with regards to genotoxicity and carcinogenicity. The critical effect observed in animal studies was maternal toxicity (gastrointestinal disturbances) in the prenatal developmental toxicity study in rabbits. The NOAEL for this effect was 500 mg advantame/kg bw/day. Advantame was well tolerated in single or repeated doses up to 0.5 mg/kg bw/day by normo-glycemic or diabetic subjects. The Panel established an ADI of 5 mg/kg bw/day based on the application of a 100-fold uncertainty factor to the NOAEL of 500 mg/kg bw/day for maternal toxicity from the prenatal developmental toxicity study in the rabbit. Conservative estimates of advantame exposure for high level adults and children consumers were below the ADI for the proposed use levels.



## KEY WORDS

food additive, intense sweetener, advantame, ANS980, ANS9801-acid, CAS Registry Number 714229-20-6

[1] On request from the European Commission, Question No EFSA-Q-2010-00943, adopted on 3 July 2013.

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## SUMMARY

Following a request from the European Commission, the Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to provide a scientific opinion on the safety of advantame as a high-intensity sweetener.

Advantame (ANS9801) is described by the applicant as a derivative of aspartame and it is reported to be approximately 37 000 times sweeter than sucrose.

The final advantame product is described as having a minimum purity of 97 % (on an anhydrous basis). The Panel noted that in several non-consecutive batches of the final product, the levels of platinum and palladium, residues from the catalysts used in the catalytic hydrogenation process, could amount to 1.7 and 5.3 mg/kg, respectively, and therefore, the Panel considered that a maximum limit for palladium and for platinum should be included in the specifications.

The advantame bulk material has been demonstrated to be stable following storage for up to five years under normal conditions and for up to six months under accelerated conditions. The stability of advantame has also been demonstrated under intended conditions of use in foods and beverages. In heat-treated beverages, it is reported that about 50 % of advantame is degraded. A similar result has been reported in yellow cake, where approximately 39 % of advantame is degraded during batter preparation and baking, and production of *N*-[*N*-[3-(3-hydroxy-4-methoxyphenyl)propyl]-α-aspartyl]-L-phenylalanine (ANS9801-acid) occurs. The Panel noted that there was an indication of advantame instability in acidic beverages and thermally treated foods.

The metabolism and toxicokinetics studies show that orally administered advantame is rapidly converted in the gastrointestinal tract to ANS9801-acid which can be rapidly absorbed. However, the bioavailability of the ANS9801-acid is limited. Following i.v. administration, advantame is rapidly converted to the ANS9801-acid. Following oral dosing of radiolabelled advantame, radioactivity is excreted mainly in the faeces, with urinary excretion representing a minor route. The ANS9801-acid metabolite has been detected in rat and human plasma. In contrast, in dogs there is little free ANS9801-acid in plasma. There was no evidence of accumulation of ANS9801-acid in healthy and type II diabetes subjects after 4 or 12 weeks of consumption. This is consistent with the short estimated half-lives of radioactivity of ANS9801-acid from the preclinical and clinical studies. ANS9801-acid can be hydrolysed to phenylalanine and an aspartic acid derivative, *N*-(3-(3-hydroxy-4-methoxyphenyl))propyl-L-aspartic acid (HF-1). HF-1 is a minor urinary metabolite in rats, dogs and humans. HF-1 can be further metabolised to 3-(3-hydroxy-4-methoxyphenyl)-1-propylamine (HU-1), which is also postulated to be formed directly from ANS9801-acid. HU-1 is a minor urinary metabolite in rats, dogs and humans, which has not been isolated from the faeces of any species. In human and dogs, HF-1 was the major faecal metabolite, however in rats the demethylated ANS9801-acid was the major faecal metabolite. Other minor urinary and faecal metabolites have been detected but not characterised. Studies were only carried out with advantame radiolabelled on the 3-hydroxy-4-methoxy-phenyl moiety of the molecule, and whilst the metabolic fate of the remainder of the molecule could not be investigated, it could be predicted. The applicant concluded that the rat is a suitable metabolic model for systemic metabolism of advantame in human. They further concluded that effects due to differences in faecal metabolites between rats and human would have been addressed by studies in dogs where faecal metabolites are similar to those in human. The Panel agreed that the experimental metabolic data supported the use of the available toxicological studies in rat and dogs for the safety assessment of advantame in humans.

In 13-week dietary studies in mice and rats, a chronic toxicity and carcinogenicity study in rats and a subchronic toxicity study in dogs, the rate and extent of systemic exposure of animals to advantame and ANS-9801 acid were highly variable and appeared to be characterised by non-linear kinetics and with no clear underlying mechanism over the dietary concentration range used in these experiments. By considering the changes in $AUC_{24}$ data in the course of these subchronic or chronic studies, the

Panel considered that advantame or ANS9801-acid do not accumulate even in case of high dietary doses given to these animal species for periods as long as 52 or 104 weeks.

Toxicokinetic data derived from a prenatal developmental toxicity study in rabbits demonstrated that when advantame administered by gavage at 2000 mg/kg bw/day, a dose similar to that achieved at advantame dietary concentration of 50 000 mg/kg diet in subchronic toxicity study in dogs and chronic toxicity and carcinogenicity study in rats, the systemic exposure to both advantame and ANS9801-acid is 5 to 25 times higher than in dietary administered animals. This would represent a worst case situation for a maximal systemic exposure of animals to advantame and ANS9801-acid. The Panel also noted that in this study, advantame and ANS-9801-acid were described to accumulate at the higher doses since increasing the dose of advantame resulted in a disproportionately higher systemic exposure to advantame than would be predicted from a linear relationship.

Overall, the Panel considered that the high variability and the lack of clear underlying mechanism did not permit such a clear conclusion on accumulation or non-linearity in kinetics

The safety of advantame has been examined in *in vitro* studies and in sub-acute, subchronic and long-term studies in mice, rats and dogs and in reproductive and developmental studies in rats and rabbits.

Since the $LD_{50}$ for advantame in rats was shown to be higher than 5000 mg/kg bw in an oral rat study there is no concern with respect to the acute toxicity of advantame.

The Panel considered these changes as treatment related as food consumption in treated groups was comparable to controls but decreases in food efficiency were recorded and several haematological, blood chemistry and urinalysis parameters were also statistically significantly altered at high dietary concentrations of advantame. At lower dose levels the recorded changes

In all of the subchronic toxicity studies apart from the 13-week study in rats and 1-year study in dog decreases in body weight/body weight gain were seen at a dietary concentration of 50 000 mg advantame/kg diet. The Panel considered these changes as treatment related as food consumption in treated groups was comparable to controls but decreases in food efficiency were recorded and several haematological, blood chemistry and urinalysis parameters were also statistically significantly altered at high dietary concentrations of advantame. At lower dose levels the recorded changes, although statistically significant, were often slight and within the physiological ranges for the species, a dose-response was not apparent, they were not accompanied by morphological changes in any organs, and there was no consistency between the sexes in the recorded changes, or between the studies in the same species or between the species.

The Panel noted the changes in several parameters, indicative of an altered function of the immune system in the 13-week study in rats. The Panel noted that the repeated observation of effects on thymus and lymphocytes throughout the different subchronic studies in rats and dogs may be indicative of an effect on the immune system following exposure of laboratory animals to high dietary doses of advantame.

An additional study (TNO Triskelion report, 2013), investigating the potential immunotoxic effects of dietary administered advantame in young male and female Wistar rats, was submitted to EFSA. There were no treatment-related differences in total or differential white blood cell counts or in total protein concentration, albumin concentration and albumin/globulin ratio. There were no treatment-related differences in lymphocyte subsets. No treatment-related effects on proliferative response of splenocytes to mitogen stimulation by Con A were observed. There were no treatment-related differences in any cytokine production after mitogen stimulation. The weights of the adrenals, spleen and thymus at the end of treatment and the recovery period were not affected by treatment. Macroscopic examination and microscopic examination of lymphoid organs at the end of the treatment did not reveal any treatment related findings. The Panel considered that this study did not reveal any immunotoxic effects of advantame on the endpoints that were examined in the rat. The Panel noted

that the data provided evidence for an absence of immunotoxic effects of advantame as were suggested by the previous rat 13-week study.

Advantame was found to be not genotoxic in the bacterial reverse mutation assay, the *in vitro* mouse lymphoma TK assay and the *in vivo* mouse micronucleus assay.

In the carcinogenicity study in mice, the malignant tumours with statistically significantly increased incidences were bronchio-alveolar adenocarcinoma and histiocytic sarcoma of the haematopoietic system. The pulmonary tumours are known to have a high background incidence in mice. For both tumours there was no dose-response relationship in either sex and their incidences were within historical control ranges for CD-1 mice, thus the Panel considered that these tumours were not treatment-related. Advantame was not considered by the Panel to be carcinogenic to mice at doses up to 50 000 mg/kg diet, the highest concentration tested, and equal to 5693 and 7351 mg/kg bw/day in males and females, respectively. The Panel noted that at the high dose the body weight gain of females was significantly lower than controls and a trend toward lower body weight was also observed for male mice, however the difference did not achieve statistical significance. With regard to the lower body weight gain in the high-dose group the no observed adverse effect level (NOAEL) was 10 000 mg advantame/kg diet, equal to 1057 and 1343 mg advantame/kg bw/day in males and females, respectively.

In the carcinogenicity study in rats a higher incidence of pancreatic islet-cell carcinoma or of pancreatic islet-cell carcinoma and adenoma combined was seen in high-dose males but the incidence was not statistically significantly different from that in controls and these pancreatic tumour incidences were within the historical control values. The incidence of mammary gland adenoma in high-dose females was statistically significantly higher than that of controls but it was within the range of the historical control values. In contrast, the incidence of mammary adenocarcinoma was not statistically significantly increased in any of the treated groups. Combining the incidence of mammary gland adenomas and adenocarcinomas no pairwise comparisons achieved statistical significance. When the authors of the study combined the incidence of mammary gland adenomas, adenocarcinomas and fibroadenomas a positive trend was noted with dose but no pairwise comparisons achieved statistical significance. The trend test was no longer statistically significant upon exclusion of the high-dose group. Similar results were described by the authors for combined incidences of adenomas and fibroadenomas. Since the incidence rates of all mammary tumour types and combinations were within the historical control range, and as mammary tumours are part of the background pathology of the ageing female rat, the Panel considered the increased incidences of mammary tumours in this study to be unrelated to treatment. Consequently, the Panel considered that the neoplastic findings in the carcinogenicity studies in rats do not provide evidence of carcinogenicity of advantame tested at dietary concentrations of 50 000 mg/kg diet, the highest dose tested. This dietary concentration was equal to 2621 or 3454 mg/kg bw/day in male and female rats respectively. The Panel noted that at the high dose body weight gains in the males and females treated for 104 weeks were reduced, attaining statistical significance in males only.

Reproductive and developmental toxicity studies on advantame included a 2-generation reproduction toxicity study in rats, and prenatal developmental studies in rats and rabbits. The NOAEL for reproductive toxicity in 2-generation reproduction toxicity study in rats was 50 000 mg advantame/kg diet, the highest dose tested. The NOAEL in rats for maternal toxicity from the prenatal developmental study was 15 000 mg advantame/kg diet (equal to 1419 mg/kg bw/day) and for prenatal developmental effects was 50 000 mg advantame/kg diet/day (equal to 4828 mg/kg bw/day) the highest dose tested. In rabbits the NOAEL for maternal toxicity was 500 mg advantame/kg bw/day based on disturbances of gastrointestinal tract causing morbidity that required killing the animals for welfare reasons at the next highest dose (1000 mg/kg bw/day) and the NOAEL for developmental toxicity was 1000 mg advantame/kg bw/day.

In other special studies conducted in rats, no behavioural or physiological alterations were observed and no mortality resulted from the administration of advantame. In addition, the locomotor activity of

rats was unaffected by the administration of advantame compared with vehicle-treated animals. Although no significant effects were reported on the gastrointestinal motility of rats treated with 10 or 100 mg advantame/kg bw, a dose of 1000 mg/kg bw resulted in a moderate statistically significant decrease in gastrointestinal motility compared to rats administered the vehicle. Studies in dogs showed that the duodenal administration of advantame resulted in no significant effects on general respiratory status, resistance of the peripheral vasculature, and or on electrical status of the myocardium compared with the vehicle control group. Intra-duodenal administration of advantame produced no biologically meaningful effects on cardio-respiratory parameters.

The human tolerability of advantame has been tested in three clinical studies. Advantame was well tolerated in single doses up to 0.5 mg/kg bw or repeated doses up to 0.5 mg/kg bw/day, in healthy volunteers and in both normo-glycemic individuals and diabetics. Advantame did not affect the levels of various biochemical, haematological, or urinalysis endpoints and did not affect plasma levels of glucose or insulin in normo-glycemic individuals, or alter glucose tolerance or insulin resistance in the diabetic subjects provided advantame.

The degradation products of advantame β-ANS9801, β-ANS9801-acid, ANS9801-imide, and HF-1 have not been identified as impurities of the final advantame product. The safety of these degradation products was assessed individually in a series of *in vitro* genotoxicity assays and in an *in vivo* mouse micronucleus assay. Based on these data the Panel concluded that there is no concern with respect to genotoxicity of the degradation products of advantame. The Panel has previously reviewed data on phenylalanine and methanol concluding that they would not be of safety concern at levels greater than could be released from advantame.

Since advantame is a secondary amine, its potential to participate in the formation of *N*-nitroso compounds in foods and beverages and in the stomach was evaluated. No nitrosation of advantame was identified by the applicant and, therefore, the Panel considered that the potential formation of *N*-nitroso advantame does not raise a safety concern

After consideration of all the data on advantame and its metabolites and major and minor degradation products available for this evaluation, the Panel concluded that there were sufficient data with which to establish an acceptable daily intake (ADI).

Following specific studies the prior concern about possible immunological effects (as discussed above) other critical studies considered in setting the ADI were those indicating body weight effects in laboratory animals and those on developmental toxicity with regard to maternal toxicity and gastrointestinal symptoms in the rabbit.

The Panel considered the findings in the prematurely sacrificed rabbits from the prenatal developmental study to be related to advantame exposure. The Panel noted that although only one animal was affected in the mid-dose group (1000 mg advantame/kg bw/day), the effects observed in this animal were consistent with those found in sacrificed animals from the higher dose group (2000 mg advantame/kg bw/day). The Panel considered that the single incidence of adverse effects in the mid-dose group may be indicative of the proximity of the boundary dose for adverse effects of advantame, since incidence increased in the higher dose group. Taking into account that effects indicative of maternal toxicity in form of decreased feed intake–were also observed in the developmental toxicity study in rats upon administration of advantame through the diet, the Panel considered that effects observed in the rabbits cannot be disregarded. Therefore the Panel identified a NOAEL of 500 mg advantame/kg bw/day for maternal toxicity in the prenatal developmental toxicity study in NZW rabbits by oral gavage.

This NOAEL was further supported by the difference in the toxicokinetics of high doses of advantame (around 2000 mg/kg bw/day) in the subchronic and chronic dietary administration studies in mice, rats and dogs and the oral gavage administration in reproductive and prenatal developmental toxicity studies in the rabbit. The internal doses of advantame and ANS9801-acid as estimated by $AUC_{24}$ were

markedly higher following oral gavage administration in the rabbit compared to dietary administration in rat, mouse and dog. There were no high dose kinetic studies by gavage available for rat, mouse and dog nor were high dose dietary kinetic data available for the rabbit. Therefore the Panel was unable to ascertain whether the observed differences in internal dose were due to the route of administration or were species specific. However given the similarity of kinetics at low bolus doses in all species studied, including humans, the Panel considered that it would not be appropriate, on the available information, to disregard the oral gavage study because of the apparent increase in internal dose. Therefore, the Panel considered that the NOAEL of 500 mg advantame/kg bw/day for maternal toxicity in the rabbit developmental toxicity study should be used as the point of departure for the derivation of the ADI.

The Panel noted that either a default uncertainty factor of 100 or lower uncertainty factor based on inter-species allometric scaling and inter-human variability could be applied to account for inter-species and inter-human variability in maternal toxicity. However, the Panel considered that the more conservative of these uncertainty factors would be appropriate in this case, due to the marked variability in the internal doses arising following dietary or bolus administration which was seen at both high and low dose levels, the absence of complete kinetic data set (high and low dose with both methods of administration) in any species studied and the corresponding difficulty in defining a clear kinetic model.

Anticipated exposures to advantame from its proposed use as a food additive and its metabolites (methanol and phenylalanine) have been calculated (mean and 95$^{th}$ percentile of consumers only) using the food consumption data at the individual level (e.g. raw data on food consumption by the individual consumer).

High-level consumption was only calculated for those foods and population groups where the sample size was sufficiently large to allow calculation of the 95$^{th}$ percentile. The Panel estimated chronic exposure for the following population groups: toddlers, children, adolescents, adults and the elderly. Calculations were performed using individual body weights.

Thus, for the present assessment, food consumption data were available from 26 different dietary surveys carried out in 17 different European countries.

When considering the proposed maximum use levels, the mean dietary exposure to advantame in European children-adolescents (aged 1-17 years) ranged from 0.02 to 0.33 mg/kg bw/day, and from 0.05 to 0.74 mg/kg bw/day for high level consumers (95$^{th}$ percentile). Exposure estimates for the European adult population give a mean dietary exposure to advantame ranged from 0.01-0.12 mg/kg bw/day, and from 0.03-0.28 mg/kg bw/day for high level consumers (95$^{th}$ percentile).

The Panel noted that its estimates could be considered as being conservative as it was assumed that all processed foods and beverages contained the sweetener advantame added at the maximum proposed use levels. It has to be noted that the anticipated exposure to advantame did not include possible applications from its use as an excipient in pharmaceutical products, an application which might occur if advantame were an approved food additive.

The exposure to methanol, which may result from ingestion of advantame-containing foods and beverages, was considered negligible compared to that from other dietary sources and as such of no concern from the safety point of view.

The exposure to phenylalanine expected from ingestion of advantame as a general purpose sweetener was considered of no safety concern for healthy consumers (adults and children). For a phenylketonuric child, the additional phenylalanine intake expected from ingestion of advantame-containing foods and beverages would represent a relatively small increment in the exposure to phenylalanine.

After considering all the data available, the Panel concluded that advantame does not raise concern with regards to genotoxicity and carcinogenicity. The critical effect observed in animal studies was maternal toxicity (gastrointestinal disturbances) in the prenatal developmental toxicity study in rabbits. The NOAEL for this effect was 500 mg advantame/kg bw/day.

The Panel established an ADI of 5 mg/kg bw/day based on the application of a 100-fold uncertainty factor to the NOAEL of 500 mg advantame/kg bw/day for maternal toxicity from the prenatal developmental toxicity study in the rabbit.

Conservative estimates of advantame exposure for high level adults and children consumers were below the ADI for the proposed use levels.

After considering all the data on stability, degradation products, toxicology and exposure, the Panel concluded that advantame would not be of safety concern at the proposed uses and use levels as a sweetener.

The Panel considered that a maximum limit for palladium and for platinum should be included in the specifications.

## TABLE OF CONTENTS

## BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The use of food additives is regulated under European Parliament and Council Regulation (EC) No 1333/2008 on Food additives. Only food additives that will be included in the Community list in Annex II of that regulation may be placed on the market and used in foods under the conditions of use specified therein.

A manufacturer has requested the authorisation of a new sweetener (so-called advantame) in a number of food categories.

## TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (I) (a) of Regulation (EC) No 178/2002, the European Commission asks the European Food Safety Authority to provide a scientific opinion on the safety advantame when used as a food additive in the food categories specified in the application dossier.

## ASSESSMENT

### 1. Introduction

The present opinion deals with the safety of advantame when used as a high-intensity sweetener in various food and tabletop products.

Advantame is described by the applicant as a derivative of aspartame. It is reported to be approximately 37 000 times sweeter than sucrose.

### 2. Technical data

### 2.1. Identity of the substance

Advantame (ANS9801), is described by the applicant as an *N*-substituted (aspartic acid portion) derivative of aspartame.

The molecular formula of advantame monohydrate is $C_{24}H_{30}N_2O_7 \cdot H_2O$ and it has a molecular weight of 476.5 g/mol (monohydrate). Its CAS Registry Number is 714229-20-6 and the IUPAC name is *N*-[*N*-[3-(3-hydroxy-4-methoxyphenyl)propyl]-α-aspartyl]-L-phenylalanine 1-methyl ester, monohydrate. Figure 1 shows the structural formula.

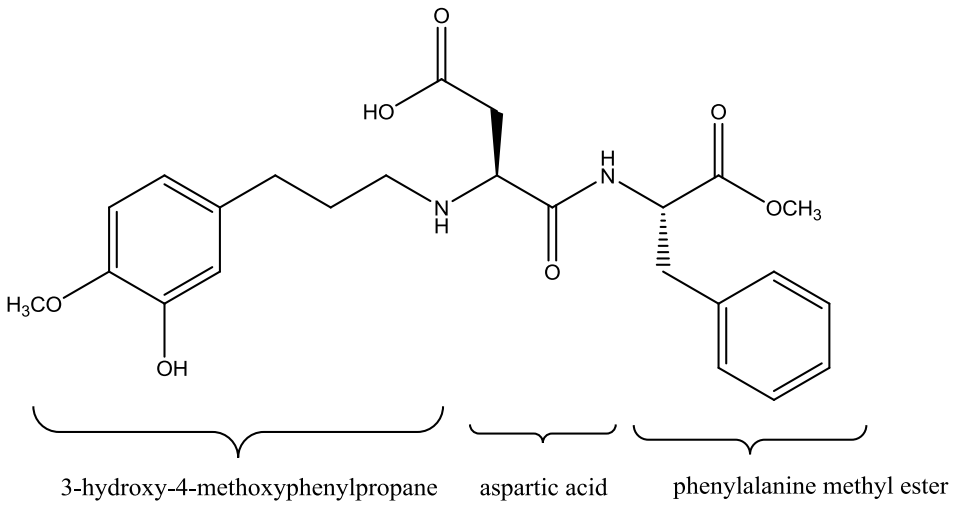



**Figure 1:** Structural formula of advantame

Advantame is proposed as the common or usual name for the compound.

Synonyms are: (3*R*)-4-[[(1*S*)-1-benzyl-2-methoxy-2-oxo-ethyl]amino]-3-[3-(3-hydroxy-4-methoxy-phenyl)propylamino]-4-oxo-butanoic acid hydrate; (3*R*)-4-[[(1*S*)-1-benzyl-2-methoxy-2-oxo-ethyl]amino]-3-[3-(3-hydroxy-4-methoxyphenyl)propylamino]-4-oxo-butanoic acid hydrate; (*R*)-3-[3-(3-hydroxy-4-methoxyphenyl)propylamino]-4-[(*S*)-1-methoxy-1-oxo-3-phenylpropan-2-ylamino]-4-oxobutanoic acid hydrate; aminosweet.

The solubility of advantame in water is 0.99 g/l at 25 °C with increasing solubility at higher temperatures and lower pH.

### 2.2. Specifications

Advantame is described by the applicant as a powder, which can vary in colour from white to yellow with a melting point of 101.5 °C. The final advantame product is described to have a minimum purity of 97 % purity (on an anhydrous basis).

According to the applicant, the final product, advantame, was analysed for the presence of heavy metals (i.e. lead and arsenic), residual solvents (methanol and isopropyl acetate used in the preparation of advantame; methyl acetate and 2-propanol that could be formed by reaction and hydrolysis of the previous mentioned solvents), residual hydrogenation catalysts (i.e., palladium on aluminium oxide and platinum on carbon) used in the manufacturing process, and substances structurally related to advantame formed during the preparation of advantame. The Panel noted that no analytical data were provided for the presence of the potential intermediate compound 3-hydroxy-4-methoxycinnamaldehyde (HMCA) in the final product. The applicant stated that it is unlikely that a significant amount of this substance would be present. Analysis for several potential intermediate compounds, including 3-(3-hydroxy-4-methoxyphenyl)propionaldehyde (HMPA), di-HMPA and HMPA-alcohol, resulting from the manufacturing process were reported but the substances were not present at detectable levels in any of the batches analysed.

A total of 27 related substances were identified in the final product using high-performance liquid chromatography (HPLC). The majority of them were individually detectable between 0.01 and 0.02 % and the total content of the related substances ranged from 0.6 to 1.4 % in the final product. Among them 16 peaks of some related substances were identified in amounts that were quantifiable but 11 were identified consistently in very small quantities (< 0.1 % in the final product). Five related substances, present in the final product at levels > 0.1 % (up to 0.41 %), were identified as the following:

- α-aspartyl-L-phenylalanine 1-methyl ester ***(aspartame)***;
- *N*-[*N*-[3-(3-hydroxy-4-methoxyphenyl)propyl]-α-aspartyl]-L-phenylalanine (***ANS9801-acid***). The ANS9801-acid, apart from being a degradation product of advantame, is also its main metabolite (see Figure 2)
- *N*-[*N*-[3-(3-hydroxy-4-methoxyphenyl)pentyl]-α-L-aspartyl]-L-phenylalanine 1-methyl ester (***9801-D***);
- *N*-[*N*-[3-(3-hydroxy-4-methoxyphenyl)heptyl]-α-L-aspartyl]-L-phenylalanine 1-methyl ester (***9801-T***); and
- *N*-[*N*-[*N*-[3-(3-hydroxy-4-methoxyphenyl)propyl]-α-L-aspartyl]-α-L-aspartyl]-L-phenylalanine 1-methyl ester (***N-Alkyl-AAPM***).

Table 1 shows a summary of the specifications for advantame as proposed by the applicant (Application dossier, 2010).

**Table 1:** Specifications for advantame as proposed by the applicant (Application dossier, 2010)

| Specification Parameter | Specification Value |
|---|---|
| **Identification** | |
| Description | White to yellow powder |
| **Purity** | |
| Assay | ≥ 97.0 % and ≤ 102.0 % on anhydrous basis |
| *N*-[*N*-[3-(3-hydroxy-4-methoxyphenyl)propyl-α-aspartyl]-L-phenylalanine (ANS9801-acid) | ≤ 1.0 % |
| Total other related substances | ≤ 1.5 % |
| Water | ≤ 5.0 % |
| Residue on ignition | ≤ 0.2 % |
| Lead | ≤ 1 mg/kg |
| Arsenic | ≤ 2 mg/kg |
| **Residual solvents** | |
| Methyl acetate | ≤ 500 mg/kg |
| Isopropyl acetate | ≤ 2000 mg/kg |
| Methanol | ≤ 500 mg/kg |
| 2-Propanol | ≤500 mg/kg |

The Panel was provided with the results of analysis for twelve batches of advantame demonstrating the compliance of the final product with the proposed specifications.

The Panel noted that in several non consecutive batches of the final product, the levels of platinum and palladium, residues from the catalysts used in the catalytic hydrogenation process, could amount up to 1.7 and 5.3 mg/kg, respectively, despite the fact that in most lots (9 out of 12) the average level of palladium was less than 0.2 mg/kg and the average level of platinum was 0.7 mg/kg. The Panel considered that a maximum limit for palladium and for platinum should be included in the specifications.

Recently, according to the information in summary and conclusions of the 77$^{th}$ JECFA meeting (issued 19 June 2013), JECFA specifications for advantame were prepared but they are not yet published (JECFA, 2013).

## 2.3. Manufacturing process

Advantame is produced by chemical synthesis. Details of the manufacturing process were available to the Panel.

According to the applicant advantame is manufactured in a three-step process; production of the principal manufacturing intermediate, HMCA, followed by the hydrogenation to form HMPA. In the final step, the HMPA methanol solution (filtrate) is combined with aspartame to give the imine that under selective hydrogenation forms advantame. The solution is allowed to crystallise and crude crystals are washed. The product is re-crystallised and crystals are separated, washed and dried.

According to the applicant, in the production process development, HMPA and its related substances (HMPA-alcohol and di-HMPA) were assayed, however, these were not evaluated in the batch analysis since, theoretically, it is unlikely that a significant amount of these substances remains in the final product.

## 2.4. Methods of analysis in foods

The applicant described methods for the analysis of advantame and ANS9801-acid in various food matrices using slightly different approaches depending on the complexity of the matrix. Tabletop products, and powdered soft drink mixes could be dissolved and analysed directly, whilst carbonated soft drinks needed to be degassed. Batters, cakes and yoghurt were solvent extracted, filtered and then subjected to clean-up by cation-exchange solid phase extraction (SPE). Chewing gum was dissolved in toluene, extracted and directly analysed. In all cases HPLC with UV detection was employed giving adequate specificity based on spiking of blank samples. These methods were validated and appeared to perform adequately with well-defined food systems. However, the Panel considered that with unknown samples greater specificity in detection would probably be required.

## 2.5. Stability, reaction and fate in food

The applicant described a series of studies to evaluate the stability of advantame when stored in bulk material as well as in food and beverage matrices. Advantame in dry form is reported to be stable under standard conditions of storage (25 °C/60 % RH) for up to 60 months. Advantame was also shown to remain stable under stress conditions (accelerated stability testing, i.e. 40 °C/75 % RH) for up to six months. In addition, the applicant evaluated the photostability of advantame and reported that exposure to light during one and two weeks of testing demonstrated that advantame dry powder is stable against light.

The stability of advantame in food was tested in powdered preparations (tabletop sweetener product and dry powder beverage mix), carbonated drinks (non-aseptically processed), heat-treated beverages, yellow cake, yogurt, and chewing gum, in order to cover most pH, temperature, and moisture extremes that the ingredient might be subjected to when added to the various food and beverage products under the proposed conditions of use. For each food matrix, the stability was assessed by analysis for levels of advantame and in most cases (with the exception of yogurt), ANS9801-acid content. In carbonated soft drinks, levels of other related substances, β-ANS9801 and β-ANS9801-acid, also were measured.

The stability of advantame in powder form was tested for 36 months at 25 ± 2 °C and 60 % RH; the compound was stable but only an increase (12 %) of ANS9801-acid content was found. The analysis of advantame in lemon-flavoured dry powdered beverage stored at normal conditions (25 ± 2 °C /60 % RH) demonstrated that 96 % of the initial advantame remained intact after 12 months and 93 % after six months under accelerated conditions (40 ± 2 °C/75 % RH).

Examination of advantame stability in hot-packed flavoured beverages aimed to evaluate the stability before pasteurization and after the hot-pack process. The hot-packed flavoured beverages were formulated to have a pH of approximately 3.2. Formulated beverages were subjected to pasteurization by controlling the temperature of the beverage at the outlet if the plate heat exchanger to 96 ± 2 °C and were subsequently hot packed in glass bottles. Stability and functionality were analysed for up to 26 weeks of standard storage conditions. The results showed that short heat treatment caused less than 2 % degradation of advantame, whereas following the 26-week storage period, approximately 50 % of advantame degraded, accompanied by an increase in ANS9801-acid (i.e., from 0.15 µg/ml at baseline to 1.28 µg/ml at week 26). As approximately 16 % of the degraded advantame is unaccounted for as ANS9801-acid, the applicant speculates that degradation products of advantame were not sufficiently recovered from the ion-exchange purification step prior to the analysis.

Similar degradation of advantame (approximately 50 % of the baseline content) was demonstrated in carbonated soft drinks (matrix pH 3.2) after a 26-week storage period. Examined degradation products were ANS9801-acid (3.14 µg/ml), β-ANS9801 (0.32 µg/ml) and β-ANS9801-acid (0.70 µg/ml).

The stability of advantame in baked good, yellow cakes, was analysed. An assessment of the concentration of advantame was conducted on yellow cake batter before and after baking (at 180 °C for 35 minutes followed by cooling to room temperature for 30 minutes). Approximately 13.2 % of the added advantame was lost during batter preparation and approximately 25.2 % was lost during baking.

The decrease in advantame levels was accompanied by an increase in the levels of ANS9801-acid (22 % during baking). Therefore, approximately 39 % of the advantame was degraded during the full process.

The chemical stability of advantame in yogurt was evaluated following refrigerated storage conditions (5 ± 2 °C) for five weeks. It was observed that 24 % of advantame was degraded within these five weeks. Although the degradation products could not be identified (due to interference), they were expected to be ANS9801-acid and potentially *N*-(3-(3-hydroxy-4-methoxyphenyl))-propyl-L-aspartic acid (HF-1) resulting from peptide hydrolysis by the two fermentation microorganisms, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.

The stability of advantame in chewing gum was evaluated following storage for 27 weeks at 2 5 ± 2 °C/ 60 % RH. The reductions in the advantame level (< 10%) was accompanied by an increase in the level of ANS9801-acid.

The Panel noted that there was an indication of advantame instability in acidic beverages and thermally treated foods and that the main degradation products are, in equimolar quantities, ANS9801-acid and methanol (not measured by the applicant) being the other anticipated product.

A study was conducted to assess the possible degradation products of advantame in mock beverages with advantame at concentration of 50 mg/l (four times more concentrated than the anticipated use levels). Hydrolysis of advantame, resulting in the formation of ANS9801-acid and methanol, represents the major degradation pathway. The acid form of advantame is a known impurity of the final advantame product, with its presence limited to less than 1 % by the proposed specifications. In contrast, β-rearrangement comprises the minor degradation pathway. HF-1, which is also a metabolite of advantame, β-ANS9801, β-ANS9801-acid, ANS9801-imide, L-phenylalanine methyl ester and L-phenylalanine were identified as the minor degradation products of advantame.

Figure 2 shows the major and minor degradation pathways for advantame.

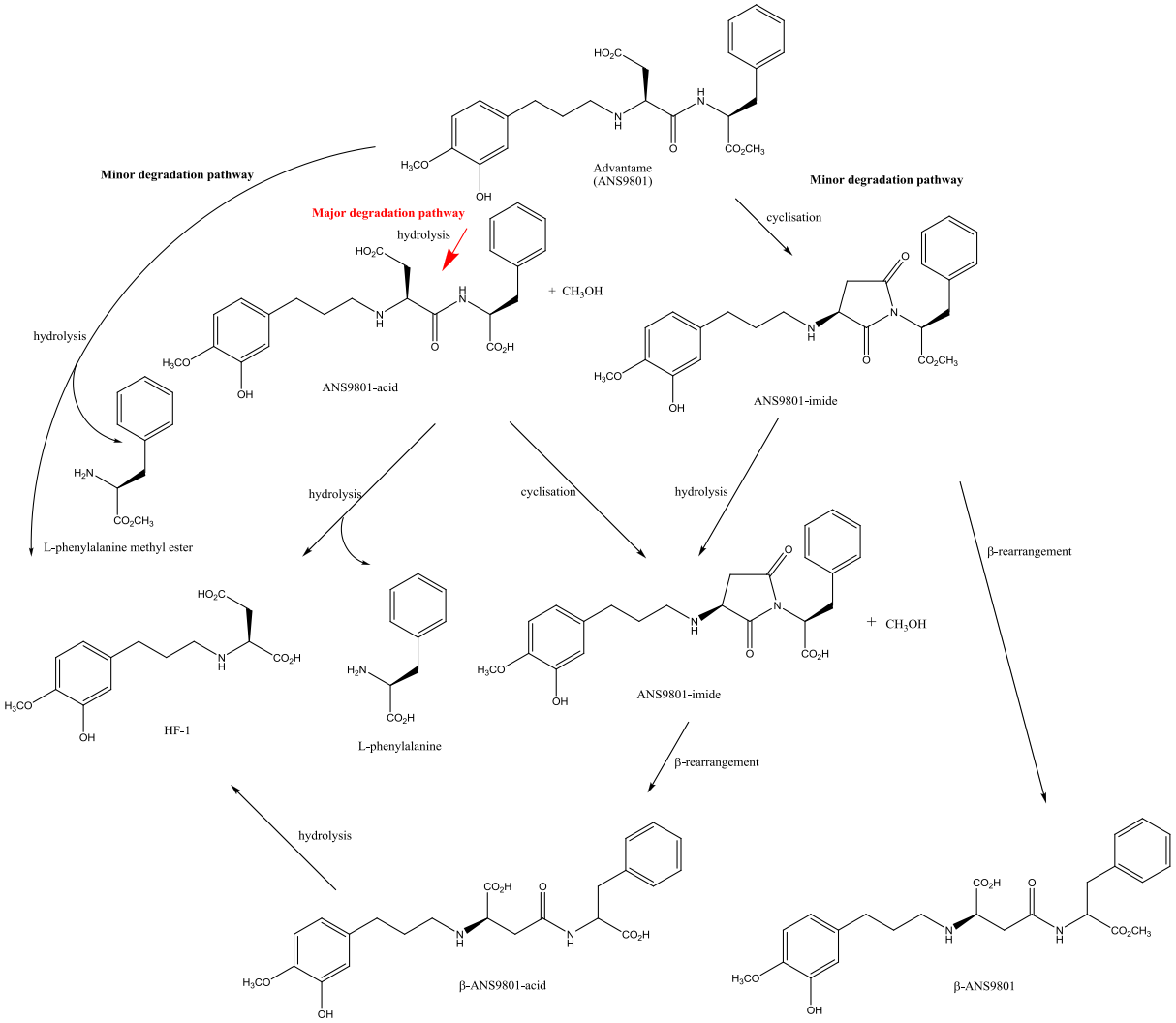



**Figure 2:** Major and minor chemical degradation pathways for advantame

The Panel noted that the secondary amine group of advantame could potentially react in a Maillard-type reaction, and that therefore interactions between food constituents and advantame are possible.

As a secondary amine, advantame may react under nitrosation conditions to form *N*-nitrosamines in some food and beverages and in the stomach. This is further discussed in the section 3.2.6.4.

In contrast to aspartame, advantame cannot cyclise to form the diketopiperazine derivative, as there is not a free amino group to start the internal reaction of cyclisation.

## 2.6. Case of need and proposed uses

The applicant proposed to use advantame as a high-intensity sweetener in the categories of foods in which high-intensity sweeteners are permitted by the current EU legislation, Annex II to Regulation (EC) No 1333/2008[4] on food additives.

Studies assessing the sweetening potency, demonstrated that advantame is approximately 37 000 times sweeter than sucrose. The food uses and use level for advantame proposed by the applicant are presented in Table 2.

[4] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008, p. 1-18.

**Table 2:** Food uses and maximum use level for advantame proposed by the applicant.

| Category number | Foods | restrictions/exception | Maximum use level proposed for advantame (mg/l or mg/kg as appropriate) |
|---|---|---|---|
| **1.4** | **Flavoured fermented milk products including heat-treated products** | only energy-reduced products or with no added sugar | **20** |
| **3** | **Edible ices** | only energy-reduced or with no added sugar | **16** |
| **4.2.2** | **Fruit and vegetables in vinegar, oil, or brine** | only sweet-sour preserves of fruit and vegetables | **6** |
| **4.2.3** | **Canned or bottled fruit and vegetables** | only fruit energy-reduced or with no added sugar | **20** |
| **4.2.4.1** | **Fruit and vegetable preparations excluding compote** | only energy-reduced | **20** |
| **4.2.5.1** | **Extra jam and extra jelly as defined by Directive 2001/113/EEC** | only energy-reduced jams jellies and marmalades | **20** |
| **4.2.5.2** | **Jam, jellies and marmalades and sweetened chestnut puree as defined by Directive 2001/113/EEC** | only energy-reduced jams, jellies and *marmalades* | **20** |
| **4.2.5.3** | **Other similar fruit or vegetable spreads** | only dried-fruit-based sandwich spreads, energy-reduced or with no added sugar | **20** |
| **5.1** | **Cocoa and Chocolate products as covered by Directive 2000/36/EC** | only energy-reduced or with no added sugars | **40** |
| **5.2** | **Other confectionery including breath refreshing microsweets** | only cocoa or dried fruit based, energy reduced or with no added sugar | **40** |
| **5.2** | **Other confectionery including breath refreshing microsweets** | only cocoa, milk, dried fruit or fat based sandwich spreads, energy-reduced or with no added sugar | **20** |
| **5.2** | **Other confectionery including breath refreshing microsweets** | only starch based confectionary energy reduced or with no added sugar | **40** |
| **5.2** | **Other confectionery including breath refreshing microsweets** | only confectionary with no added sugar | **20** |
| **5.2** | **Other confectionery including breath refreshing microsweets** | only breath-freshening micro-sweets, with no added sugar | **120** |
| **5.2** | **Other confectionery including breath refreshing microsweets** | only strongly flavoured freshening throat pastilles with no added sugar | **40** |
| **5.3** | **Chewing gum** | only with added sugars or polyols, as flavour enhancer | **200** |
| **5.3** | **Chewing gum** | only with no added sugar | **400** |

| Category number | Foods | restrictions/exception | Maximum use level proposed for advantame (mg/l or mg/kg as appropriate) |
|---|---|---|---|
| **5.4** | **Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4** | only starch based confectionary energy reduced or with no added sugar | **40** |
| **5.4** | **Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4** | only confectionary with no added sugar | **20** |
| **5.4** | **Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4** | only cocoa or dried fruit based, energy reduced or with no added sugar | **40** |
| **5.4** | **Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4** | only sauces | **7** |
| **6.3** | **Breakfast cereals** | only breakfast cereals with a fibre content of more than 15 %, and containing at least 20 % bran, energy reduced or with no added sugar | **20** |
| **7.2** | **Fine bakery wares** | only essoblaten - wafer paper | **20** |
| **7.2** | **Fine bakery wares** | only fine bakery products for special nutritional uses | **34** |
| **9.2.** | **Processed fish and fishery products including molluscs and crustaceans** | only sweet-sour semi-preserves of fish and marinades of fish, crustaceans and molluscs | **6** |
| **11.4.1** | **Table-top Sweeteners in liquid form** | | **104** |
| **11.4.2** | **Table-top Sweeteners in powder form** | | **800** |
| **11.4.3** | **Table-top Sweeteners in tablets** | | **400** |
| **12.4** | **Mustard** | | **7** |
| **12.5** | **Soups and broths** | only energy-reduced soups | **3** |
| **12.6** | **Sauces** | | **7** |
| **12.7** | **Salads and savoury based sandwich spreads** | only *Feinkostsalat* | **7** |
| **13.2** | **Dietary foods for special medical purposes defined in Directive 1999/21/EC (excluding products from food category 13.1.5)** | Products in this category can also use additives that are allowed in the corresponding food counterparts categories | **20** |
| **13.3** | **Dietary foods for weight control diets intended to replace total daily food intake** | | **16** |

| Category number | Foods | restrictions/exception | Maximum use level proposed for advantame (mg/l or mg/kg as appropriate) |
|---|---|---|---|
| | **or an individual meal (the whole or part of the total daily diet)** | | |
| **14.1.3** | **Fruit nectars as defined by Council Directive 2001/112/EC and vegetable nectars and similar products** | only energy-reduced or with no added sugar | **12** |
| **14.1.4** | **Flavoured drinks** | only energy reduced or with no added sugar | **12** |
| **14.2.1** | **Beer and malt beverages** | only alcohol-free beer or with an alcohol content not exceeding 1.2 % vol; 'Bière de table/Tafelbier/Table beer' (original wort content less than 6 %) except for 'Obergäriges Einfachbier'; Beers with a minimum acidity of 30 milli-equivalents expressed as NaOH; Brown beers of the 'oud bruin' type | **12** |
| **14.2.1** | **Beer and malt beverages** | only energy-reduced beer | **0.5** |
| **14.2.3** | **Cider and perry** | | **12** |
| **14.2.8** | **Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15 % of alcohol** | | **12** |
| **15.1** | **Potato-, cereal-, flour- or starch-based snacks** | | **10** |
| **15.2** | **Processed nuts** | | **10** |
| **16** | **Desserts excluding products covered in category 1, 3 and 4** | only energy-reduced or with no added sugar | **20** |
| **17.1** | **Food supplements supplied in a solid form including capsules and tablets and similar forms excluding chewable forms** | | **40** |
| **17.2** | **Food supplements supplied in a liquid form** | | **12** |
| **17.3** | **Food supplements supplied in a syrup-type or chewable form** | | **110** |

Considering the very specific foods in which advantame is allowed and the restrictions within each food groups, it was not possible to disaggregate the foods in the food consumption database used for this estimate. Therefore, approximations have been made and are explained in detail later in this section.

## 2.7. Information on existing authorisations and evaluations

Food Standards Australia New Zealand (FSANZ) evaluated the safety of advantame when used as an intense sweetener and established an acceptable daily intake (ADI) of 5 mg/kg bw, by applying an uncertainty factor of 100 to the no observed adverse effect level (NOAEL) of 500 mg/kg bw/day from a rabbit developmental toxicity study. The FSANZ maintained that the adverse, treatment-related findings observed in rabbits could not be discounted without additional data to show that the findings were not toxicologically relevant (FSANZ, 2010, 2011).

JECFA has recently evaluated the safety of advantame as a food additive. "*The Committee established an acceptable daily intake (ADI) of 0–5 mg/kg bw/day 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"* (JECFA, 2013).

## 2.8. Exposure assessment

The applicant provided estimates of dietary exposure of advantame based on international consumption data of other sweeteners. As these are not directly applicable to the European situation, the Panel decided to perform its own assessment.

Anticipated exposure to degradation products and to metabolites of advantame, methanol and phenylalanine is presented in the section 3.2.6.3 of the opinion.

### 2.8.1. Food consumption data used for exposure assessment

In 2010, the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) was built from existing national information on food consumption at a detailed level. Competent authorities in European countries provided EFSA with data on the level of food consumption by the individual consumer from the most recent national dietary survey in their country (cf. Guidance of EFSA 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment', EFSA, 2011a).

The food consumption data gathered at EFSA were collected by different methodologies and thus direct country-to-country comparison should be made with caution.

Anticipated exposures to advantame from its proposed use as a food additive and its related by-product compounds (methanol and phenylalanine) have been calculated (mean and 95$^{th}$ percentile of consumers only) using the food consumption data at the individual level (e.g. raw data on food consumption by the individual consumer).

High-level consumption was only calculated for those foods and population groups where the sample size was sufficiently large to allow calculation of the 95$^{th}$ percentile (EFSA, 2011a). The Panel estimated chronic exposure for the following population groups: toddlers, children, adolescents, adults and the elderly. Calculations were performed using individual body weights.

For the present assessment food consumption data were available from 26 different dietary surveys carried out in 17 different European countries, as mentioned in Table 3.

**Table 3:** Population groups considered for the exposure estimates of advantame

| Population | Age range | Countries with food consumption surveys covering more than one day |
|---|---|---|
| Toddlers | from 12 up to and including 35 months of age | Belgium, Bulgaria, Finland, Germany, Italy, Netherlands, Spain |
| Children[(a)] | from 36 months up to and including 9 years of age | Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Spain, Sweden |
| Adolescents | from 10 up to and including 17 years of age | Belgium, Cyprus, Czech Republic, Denmark, France, Germany, Italy, Latvia, Spain, Sweden |
| Adults | from 18 up to and including 64 years of age | Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Spain, Sweden, UK |
| The elderly[(a)] | Older than 65 years | Belgium, Denmark, Finland, France, Germany, Hungary, Italy |

[(a)] The terms 'children' and 'the elderly' correspond respectively to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011a).

Consumption records were codified according to the FoodEx classification system (EFSA, 2011b). Nomenclature from the FoodEx classification system has been linked to the Food Classification System as presented in the Commission Regulation (EU) No 1129/2011[5], part D, to perform exposure estimates.

In order to have an accurate selection of foods in which advantame is proposed to be used and thus an accurate exposure estimate of advantame, in addition to the FoodEx nomenclature, the original national food names (i.e. original food descriptors as written in the national surveys) were used as much as possible.

### 2.8.2. Exposure to advantame from its use as a food additive

Exposure to advantame from its proposed use as a food additive has been calculated using the levels proposed by the petitioner, as listed in Table 2, combined with national consumption data for the five population groups (Table 3). A detailed summary of total estimated exposure (using proposed use levels) per age class and survey is presented in Annex B.

Most of the food items in which advantame is proposed to be used are subject to limitations (Table 2), therefore, specific foods within the Comprehensive Database were selected. The Panel noted that its estimates should be considered as being conservative as it is assumed that all processed foods contain the sweetener advantame added at the proposed use levels.

Due to the very detailed limitations laid down in the legislation, it was not always possible to match food categories in the food consumption database.

- Fine bakery wares for special nutritional purposes are not defined by EU legislation. Some Member States have their own definition, such as bakery ware that is targeted at diabetics. As it is not the intention of the Commission to define this category under 'foods intended for particular nutritional uses' (PARNUTS), category 13, it is referred to under category 7.2, fine bakery ware in the food categorisation system[6]. Indeed, these products are not targeted at the general population; therefore, the Panel decided not to consider this category in the dietary exposure estimate for advantame in the general population.

[5] Commission Regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) N°1333/2008 of the European Parliament and of the Council establishing a Union list of food additives. OJ L 295, 12.11.2011, p. 1-177.
[6] Part D (food categories) of the Commission Regulation (EU) N°1129/2011 of 11 November 2011.

- Essoblaten-wafer paper is a specific food item in the fine bakery ware category that is not present in the FoodEx nomenclature. Since its weight is very low and its consumption most probably marginal, this food was not taken into account.

- Breakfast cereals with a fibre content of > 15% and containing ≥ 20% bran, energy-reduced or with no added sugar are a very specific kind of breakfast cereals, not consumed by children and not described as such in the consumption data nomenclature. Therefore, this food was not taken into account in the present estimate.

- The distinction between products with and without added sugar was only possible for the food groups: flavoured drinks, confectionery and chewing gums, although this was unclear for some surveys. This may result in an over/under-estimation depending on the reporting of these products in the surveys. Therefore, the whole food groups were taken into account for flavoured fermented milk products including heat-treated products, edible ices, soups and broths, fruit nectars, desserts, jams and other fruit and vegetable preparations. This may result in an over-estimation depending on the real consumption of these products within the food groups.

- The food group decorations, coatings, fillings are not described and available in the nomenclature of the consumption database; thus, this food group could not be taken into account. This resulted in a minor underestimation.

- The distinction between the form of tabletop sweeteners (tablet, powder, liquid) was not available in the EFSA Comprehensive Database. Therefore, these three food groups were considered as a whole and the highest reported use level was taken into account. This could result in a minor overestimation.

- The same applies to food supplements: no distinction between the forms of the food supplements (liquid, solid, chewable forms) is possible within the FoodEx nomenclature, therefore these three food groups were considered as a whole and the highest proposed use levels were taken into account. This represents a minor overestimation.

Table 4 summarises the estimated exposure to advantame from its proposed use as food additive of all five population groups.

**Table 4:** Summary of anticipated exposure to advantame from its use as food additive using proposed use levels in five population groups (min-max across the dietary surveys in mg/kg bw/day)

| | **Anticipated exposure to advantame** (mg/kg bw/day) | | | | |
|---|---|---|---|---|---|
| | **Toddlers (12-35 months)** | **Children (3-9 years)** | **Adolescents (10-17 years)** | **Adults (18-64 years)** | **The elderly (>65 years)** |
| Anticipated exposure using proposed use levels | | | | | |
| • Mean | 0.07-0.33 | 0.05-0.25 | 0.02-0.08 | 0.01-0.12 | 0.01-0.04 |
| • High level[(a)] | 0.24-0.74 | 0.14-0.61 | 0.05-0.23 | 0.05-0.28 | 0.03-0.10 |

[(a)] Typically 95$^{th}$ percentile of consumers only

### 2.8.3. Main food categories contributing to exposure of advantame using levels proposed by the petitioner

Table 5 presents the main contributing food categories to the total exposure and the number of surveys in which each food groups contributes to exposure at this level.

**Table 5:** Main food categories contributing to the total anticipated mean dietary exposure to advantame using proposed use levels and number of surveys in which each food category is contributing over 5 % of total exposure

| | Toddlers | Children | Adolescents | Adults | The elderly |
|---|---|---|---|---|---|
| **Food Categories** | **% contribution to total exposure (Number of surveys)[(a)]** | | | | |
| 1.4 - Flavoured fermented milk products including heat-treated products | 15-74 (7) | 5-54 (14) | 10-47 (10) | 6-48 (14) | 14-35 (7) |
| 3 - Edible ices | 5-22 (4) | 6-21 (14) | 6-29 (11) | 7-20 (9) | 5-18 (4) |
| 4.2 - Processed fruit and vegetables [(b)] | 5-13 (3) | 5-10 (9) | 6-9 (9) | 5-26 (14) | 13-46 (7) |
| 5.1 - Cocoa and chocolate products as covered by Directive 2000/36/EC | 10-17 (6) | 8-35 (15) | 9-52 (12) | 7-29 (15) | 7-22 (7) |
| 5.3.1 - Chewing gum with added sugar | - | - | 6 (1) | 5 (1) | - |
| 5.3.2 - Chewing gum without added sugar | - | 6-9 (2) | 5-11 (2) | 7-10 (2) | - |
| 16 - Desserts excluding products covered in category 1, 3 and 4 | 6-19 (3) | 5-20 (8) | 7-16 (4) | 7-12 (4) | 5-16 (3) |
| 11.4 - Table-top sweeteners | - | - | - | 5-9 (6) | 10-13 (4) |
| 12.5 - Soups and broths | - | 6-18 (2) | 6-17 (2) | 8-24 (2) | 7-17 (2) |
| 12.6 - Sauces | - | 5-8 (3) | 5-9 (8) | 5-8 (7) | 5-6 (3) |
| 12.7 - Salads and savoury based sandwich spreads | - | 8-11 (2) | 14 (1) | 7-25 (2) | - |
| 14.1.3 - Fruit nectars as defined by Council Directive 2001/112/EC and vegetable nectars and similar products | 10-56 (2) | 44 (1) | - | 20-33 (2) | 13 (1) |
| 14.1.4 - Flavoured drinks with sweeteners | 6-25 (2) | 5-15 (9) | 8-48 (2) | 5-52 (12) | 6-11 (3) |
| 14.2 - Alcoholic beverages, including alcohol-free and low-alcohol counterparts | - | - | 5 (1) | 6-14 (6) | 5-11 (3) |
| 15.1 - Potato-, cereal-, flour- or starch- based snacks | 7 (1) | 5 (2) | 5-11 (3) | 5 (1) | - |
| 17 - Food supplements | - | - | - | 5 (1) | 7-14 (2) |

[(a)] Total number of surveys may be greater than total number of countries as listed in Table 3, as some countries submitted more than one survey for a specific age range.
[(b)] This category fruits and vegetables encompasses all the foods under the 4.2 category (Table 2): fruit and vegetables in vinegar, oil, or brine, canned or bottled fruit and vegetables, fruit and vegetable preparations excluding compote, extra jam and extra jelly as defined by Directive 2001/113/EEC[7], jam, jellies and marmalades and sweetened chestnut puree as defined by Directive 2001/113/EEC, other similar fruit or vegetable spreads.

### 2.8.4. Uncertainty analysis

Uncertainties in the exposure assessment of advantame have been discussed in relevant chapters earlier in this opinion. According to the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2007), the following sources of uncertainties have been considered and summarised below.

[7] Council Directive 2001/113/EC of 20 December 2001 relating to fruit, jellies and marmalades and sweetened chestnut purée intended for human consumption. OJ L 10, 12.1.2002.

**Table 6:** Qualitative evaluation of influence of uncertainties

| Sources of uncertainties | Direction[(a)] |
|---|---|
| Consumption data: different methodologies / representativeness / under reporting / misreporting / no portion size standard | +/- |
| Extrapolation from food consumption survey of few days to estimate chronic exposure | + |
| Linkage between reported use levels and food items in the consumption database: uncertainties on which precise types of food the use levels refer. | +/- |
| Occurrence data: maximum proposed use levels within a food category | + |
| Exposure model: uncertainty in possible national differences in use levels of food categories, data set not fully representative of foods on the EU market, exposure calculations based on the maximum proposed use levels | + |

[(a)] + = uncertainty with potential to cause over-estimation of exposure; - = uncertainty with potential to cause underestimation of exposure.

The Panel noted that there was not need for a full exposure assessment of the minor degradation products, phenylalanine and methanol, since maximum possible exposure estimated based on weight basis were much lower than the dietary exposure to these compounds (section 3.2.6.3).

## 3. Biological and toxicological data

Following consumption advantame is rapidly but not completely converted to ANS9801-acid in the gut, prior to absorption. Any advantame absorbed unchanged is rapidly metabolised to ANS9801-acid in the plasma. ANS9801-acid is the major metabolite of advantame and has been identified in the plasma, urine, and faeces of all tested species (humans, dogs, and rats).

The Panel noted that a full risk characterisation of the minor degradation products, phenylalanine and methanol, was not necessary as the maximum possible exposures estimated were much lower than exposures to these compounds arising from their common dietary intake (section 3.2.6.3).

### 3.1. Absorption, distribution, metabolism and excretion

Several studies have been provided by the applicant on the pharmacokinetics and metabolism of advantame and ANS9801-acid. These studies used *in vitro* systems or *in vivo* investigations in rats, dogs, and humans. With the exception of one human study, all studies of radiolabelled advantame or ANS9801-acid used compounds uniformly labelled with $^{14}C$ in the phenyl group of the 3-hydroxy-4-methoxy-(U-$^{14}C$)-phenyl. The *in vivo* studies are summarised in the publication by Ubukata et al. (2011).

#### 3.1.1. *In vitro* studies

The stability of advantame and ANS9801-acid was assessed *in vitro* in simulated gastric and intestinal fluids (report AJO173, 2002). Advantame was found to be stable at 37 °C in gastric fluid with or without pepsin or in intestinal fluid without pancreatin, for the 120-minute incubation period. It was hydrolysed to ANS9801-acid only to a very limited extent (about 3.4 %). Incubation of advantame in simulated intestinal fluid containing pancreatin resulted in rapid hydrolysis (99.4 %) to ANS9801-acid within 5 minutes. According to the applicant, the rate of advantame hydrolysis in a simulated intestinal fluid indicates that it is likely that the majority of advantame in the GI tract will be hydrolysed to the acid which is then subsequently absorbed. *In vitro* testing of ANS9801-acid revealed that it was very stable in both simulated gastric and intestinal fluids, with > 98.0 % remaining in all cases after the 120-minute incubation period. Based on this information, the Panel noted that it is likely that advantame is mainly degraded by hydrolysis in the gut.

The extent of the *in vitro* binding of $^{14}$C-advantame to proteins in human and dog plasma and binding of $^{14}$C-ANS9801-acid to proteins in human, dog and rat plasma was measured by ultrafiltration (report AJO213, 2004). Using human plasma proteins, 81-92 % of advantame and 96-97 % of ANS9801-acid were bound to proteins across concentration ranges of 10 to 1000 and 5000 ng/ml. Using rat plasma proteins, 90-92 % of ANS9801-acid were bound to proteins across the concentration range of 10 to 10 000 ng/ml. Using dog plasma proteins, 63-65 % of advantame and 62-71 % of ANS9801-acid were bound to proteins across the concentration range 20 and 100 to 20 000 and 25 000 ng/ml. According to the authors of the study, advantame and ANS9801-acid were bound to human plasma proteins with no evidence of saturation over a 100-fold concentration range for advantame and 500-fold concentration range for ANS9801-acid. The Panel noted that this study did not identify the proteins responsible for the binding of advantame.

### 3.1.2. *In vivo* studies

#### 3.1.2.1. Animal studies

***Rat studies***

The absorption and pharmacokinetics of single oral or intravenous doses of advantame were investigated in rats (AJO/184, 2004). In this study $^{14}$C-advantame (99.9 % purity) was administered to male and female Han Wistar rats at 5 or 150 mg/kg bw by gavage (30 animals/sex/group), or intravenously at a dose of 5 mg/kg bw (33 animals/sex). Blood samples were taken at intervals up to 24 hours after dosing. Results showed that radioactivity from $^{14}$C-advantame was rapidly absorbed with peak plasma concentrations of radioactivity at 0.25 hours after the 5 mg/kg dose and between 0.5 and 0.75 hours after the 150 mg/kg dose. Advantame was rapidly hydrolysed to the ANS9801-acid equivalents. At the 5 and 150 mg/kg oral dose levels, the ANS9801-acid equivalents present in the plasma accounted for respectively 6 and 11 % of the corresponding area under the concentration-time curve (AUC) value of the total radioactivity. The terminal plasma half-life of ANS9801-acid equivalents was 2-3.6 hours after oral administration of $^{14}$C-advantame. The half-life of radioactivity after oral dosing was approximately 7 hours. Following intravenous administration, the level of ANS9801-acid equivalents present in the plasma was greater in comparison to oral gavage, accounting for approximately 37 % of the AUC. According to the authors, this may indicate that there was pre-systemic metabolism of advantame and ANS9801-acid equivalents in the rat. The oral bioavailability was reported to be approximately 8 % based on the ratio of the oral to the intravenous AUC of the total radioactivity. The Panel noted that in this study report, the major part of the plasma radioactivity was not chemically identified, particularly after oral administration of $^{14}$C-advantame.

Tissue distribution of the radioactivity was qualitatively assessed by whole body autoradiography of rats after receiving radiolabelled advantame (AJO217, 2004). Male, non-pregnant female and time-mated pregnant female Wistar rats (5/group) were administered a single oral gavage dose of 5 mg/kg bw of $^{14}$C-advantame (98.8 % purity). Animals were sacrificed at intervals up to 12 hours post dosing and examined. Data showed that male, non-pregnant female and time-mated pregnant female have similar tissue distribution and patterns of decline of the tissue radioactivity with time. Radioactivity from $^{14}$C-advantame was primarily localised in organs and tissues associated with absorption, metabolism, and excretion (mainly in gastrointestinal tract, but also in liver, kidneys and bladder) with the highest levels of radioactivity present up to 2 hours post dosing.

Another tissue distribution study was performed in order to determine the concentration and distribution of advantame in the blood and tissue of the rats (AJO181, 2002). Advantame (99 % purity) was administered to 21 male Lister Hooded rats as a single oral gavage dose of 5 mg/kg bw of $^{14}$C-advantame. Animals were sacrificed at different intervals up to 48 hours after dosing. The peak plasma radioactivity occurred 15 minutes after the administration of $^{14}$C-advantame. This declined to approximately 10 % of the peak level 6 hours after dosing. The greatest amounts of radioactivity were measured in the stomach after 15 minutes, in the small intestine after 1-2 hours, in the large intestine

after 6 hours and in the caecal contents after 12 hours. Approximately 89-97 % of the initial dose of $^{14}$C-advantame was present in the gastrointestinal tract from 0.25 to 6 hours after administration.

Metabolism and elimination of advantame were investigated in a preliminary study in which male and female Han Wistar rats were administered 5 mg/kg bw $^{14}$C-advantame (98 % purity) by gavage in five separate experiments (3-4 animals/sex/experiment) (AJO172, 2005). Absorption of radioactivity from advantame was rapid, with the peak concentration measured 0.5 hours after oral dosing; levels declined quickly, becoming undetectable 8 hours after dosing. ANS9801-acid was the predominant plasma metabolite present 0.5 hours after oral dosing, accounting for 44 and 58 % of the plasma radioactivity in males and females, respectively. The main route of elimination was via the faeces (approximately 89 % of the initial oral dose), whereas urinary and biliary excretion were minor routes (2-3 % and 5-6 % of the administered radioactivity, respectively). The metabolites identified in the urine, faeces, and bile included ANS9801-acid, demethylated ANS9801-acid (termed RF-1), and other minor metabolites. In the faeces the major metabolites were ANS9801-acid (42 % and 38 % of dose in males and females respectively) and RF-1 (20 % and 33 % of dose in males and females respectively). Three minor uncharacterised metabolites (designated as R1-R3) were also detected, each accounting for no more than 4 % of the dose. In urine, ANS9801-acid along with three minor metabolites (R1-R3) were present, with each one accounting for no more than 1 % of the administered dose.

Excretion of advantame was also investigated in a study in which single doses of $^{14}$C-advantame (98 % purity) were administered to Han Wistar rats (4/sex/dose) orally by gavage (5 or 150 mg/kg bw) or by intravenous injection (5 mg/kg bw) (AJO194, 2005). Urine and faeces were collected at intervals up to 96 hours after dosing. Results demonstrated that the radioactivity was rapidly, although poorly absorbed (4 to 8 % of an oral dose was absorbed, in a dose-dependent manner) and excreted in the urine and faeces. Urinary excretion accounted for 0.97-1.94 % of the absorbed dose while the majority of excreted radioactivity was recovered in the faeces. The main metabolite detected in both urine and faeces was ANS9801-acid (30 % and 90 % of measured radioactivity respectively within 24 hours of oral exposure to the low and high dose). Following intravenous administration, ANS9801-acid was also identified as the main urinary metabolite (20 % of the total radioactivity). Parent advantame was detected in the urine of only two animals (1/sex) 0-6 hours following intravenous administration and accounted for 1.5 % of the dose given; other metabolites identified in the urine were 3-(3-hydroxy-4-methoxyphenyl)-1-propylamine, a metabolite termed HU-1, and *N*-(3-(3-hydroxy-4-methoxyphenyl))propyl-L-aspartic acid, termed HF-1. In the faeces RF-1 (30 % of total $^{14}$C) and ANS9801-acid (20-30 % of total $^{14}$C) were the main metabolites identified following intravenous administration of advantame. A third minor unidentified component in the faeces, designated RF-2 accounted for approximately 11 % and 8 % of the dose, in males and females after oral and intravenous doses at 5 mg/kg bw, respectively.

***Dog studies***

The ADME of advantame was investigated in a preliminary study in which two groups of one dog of each sex received 5 mg/kg bw of $^{14}$C-advantame (98 % purity) either orally by gavage or intravenously (AJO180, 2005). Blood samples were collected at intervals up to 24 and 72 hours post dosing, respectively. Elimination of radioactivity in the urine and faeces was monitored over a 72-hour period. Results indicated that radioactivity from advantame was slowly absorbed in the dog after an oral dose (12 % - 23 %, for male and female, respectively), with peak plasma concentrations at approximately 6 hours after dosing. Advantame was rapidly converted to ANS9801-acid, which was the major metabolite present in the plasma at all time points, after both routes of administration. Other minor metabolites were detected in plasma and faecal samples. After oral administration 95-97 % of the radioactivity given was recovered from urine and faeces while only 73-78 % was recovered following the intravenous dose. In all cases, radioactivity was excreted rapidly, being almost entirely excreted within 24 hours after oral administration of advantame.

The main study in dogs investigated absorption, pharmacokinetics and metabolism of single oral or intravenous doses of advantame (AJO193, 2005). Beagle dogs (3/sex/dose) were administered $^{14}$C-

advantame (98 % purity) by gavage at doses of 5 or 150 mg/kg bw, or intravenously at dose of 5 mg/kg bw. After oral administration blood, urine and faeces samples were collected for up to 120 hours. Following intravenous administration, blood samples were collected at intervals up to 168 hours. Urine and faeces were collected up to 24 hours post dosing, and every 24 hours up to 168 hours post-dosing. Following oral administration, the radioactivity was absorbed slowly, with peak plasma concentrations occurring 6-8 hours post-dosing and the terminal half-life for total plasma radioactivity was 74-85 hours. Advantame and ANS9801-acid accounted for < 0.4 % of the total plasma radioactivity for all dose routes and levels. However, according to the authors, sulphate conjugates of ANS9801-acid, measured using the original methods would contribute to the majority of ANS9801-acid equivalents of radioactivity (data not presented). It was confirmed in the 52-week dog study that the sulphate conjugate of ANS9801-acid was present in dog plasma. Over the oral dose range, the pharmacokinetic parameters indicated that the kinetics of the 5 and 150 mg/kg bw doses were dose-dependent and non-linear. Oral absorption was estimated at 19-20 % or 10-13 % of the administered dose in dogs dosed orally with 5 or 150 mg $^{14}$C-advantame/kg bw, respectively. The oral bioavailability was calculated to be around 13 % based on the ratio of oral to intravenous AUC of the total radioactivity. The rate of excretion of $^{14}$C-advantame after oral and intravenous doses was biphasic, with a greater proportion of radioactivity of the administered $^{14}$C-advantame dose excreted during the first 24 hours. Approximately 7 % of the dose at 5 mg/kg bw was excreted via the urine and 82-85 % was found in the faeces. The radioactive metabolite profiles of faecal and urine samples were qualitatively similar across all dose groups. ANS9801-acid accounted for 1.6-3 % and 70-78 % of the dose in urine and faeces, respectively while HF-1 and HU-1 represented 1-4 % of the dose in these excreta.

Tissue distribution and elimination studies were performed in an additional study involving four male beagle dogs who received a single oral gavage dose of 5 mg $^{14}$C-advantame/kg bw (98.6 % purity) and were sacrificed at either 6, 72, 144 or 288 hours post-dosing (AJO191, 2002). Blood was collected at intervals up to 24 hours after dosing, and at 24-hour intervals up to the sacrifice. Data showed that tissue radioactivity was low, especially in some tissues (i.e., eyes, brain, and spinal cord). The concentration of radioactivity was greatest in the bile at the 6-hour sampling point representing approximately 0.1 % of the dose. The concentrations of the radioactivity in the plasma generally exceeded that in the tissues at all time points, with the exception of bile and the large intestine at the 6-hour sampling time. The mean half-life of the plasma radioactivity was 71 hours and the rate of decline of radioactivity was similar for most tissues measured.

#### 3.1.2.2. Human studies

In an initial study, three groups of 8 male subjects (18-40 years) received a single oral dose of advantame in drinking water to provide 0.1, 0.25 or 0.5 mg/kg bw for groups 1, 2 or 3 respectively (ANSE-101, 2003). A total of 743 plasma samples were analysed for plasma concentrations of advantame and ANS9801-acid, using a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method. Plasma concentrations for advantame were several orders lower than for ANS9801-acid. Since plasma concentrations of the unchanged advantame were below the limit of quantification of the assay (LOQ < 0.5 ng/ml) at all time points in all subjects administered 0.1 and 0.25 mg advantame/kg bw, the pharmacokinetic analysis of the advantame plasma concentration-time data was not possible for these groups. Only limited data were available for advantame following administration of the 0.5 mg/kg bw dose. Increasing the dose of advantame resulted in increased peak plasma concentrations of ANS9801-acid and AUC of this metabolite. According to the authors of the study, these increases appeared to be proportionate to the dose, therefore ANS9801-acid appeared to exhibit dose-independent kinetics in healthy male subjects administered single oral doses of advantame over the dose range 0.1-0.5 mg/kg bw. In contrast, the terminal half-life of ANS9801-acid increased with an increase in dose (3.6, 6.0 and 10.9 hours for doses of 0.1, 0.25 or 0.5 mg/kg bw, respectively). The authors suggested that as the terminal half-life was measured over different time periods for the different doses, a comparison of this parameter across the dose range was not appropriate.

A second study evaluated the absorption, pharmacokinetics, metabolism and excretion of a single oral nominal dose of 18.75 mg $^{14}$C-advantame (approximately 0.25 mg/kg bw) in aqueous solution (AJO210, 2005). Following an overnight fast, six healthy male subjects ingested the test article in approximately 150 ml of water followed by 150 ml of water to rinse the dosing vessel.

Blood was sampled from the subjects before dosing and at timed intervals up to 168 hours post-dose. All urine and faeces samples were collected between 12 hours before dosing and at intervals up to 168 hours post-dosing. The blood, plasma, urine and faeces were analysed for radioactivity by liquid scintillation counting either directly or after combustion of the samples. Plasma was analysed for advantame and ANS9801-acid by LC-MS/MS. Radioactive metabolites were separated from urine and faeces by extraction and chromatography with radio-detection.

Radioactivity from $^{14}$C-advantame was rapidly, but poorly absorbed. Advantame was detected in only isolated samples of plasma taken from subjects early in the sampling schedule. This confirmed that advantame was rapidly metabolised. Mean ANS9801-acid peaked at 1.75 hours after dosing. The decline of mean plasma concentrations of ANS9801-acid was characterized by an elimination half-life of 5.7 hours. The proportion of ANS9801-acid in the total plasma radioactivity was approximately 72 % and this proportion was maintained over time. ANS9801-acid represented 92.0 % of plasma radioactivity on comparison of AUC values. This suggested that only very small amounts of other metabolites would be present in human plasma.

Excretion of radioactivity was essentially complete by 120 hours after dosing with 6.2 % and 89.5 % of the dose excreted in urine and faeces, respectively. On average 84 % of the total urinary excretion of radioactivity had been excreted by 48 hours post-dosing. Radioactive metabolites in urine and faeces extracts were separated and measured by HPLC with radio-detection and no unchanged advantame was detected in any samples.

Analysis of the majority of urinary radioactivity (89 %) showed that ANS9801-acid was the predominant metabolite detected in urine collected at the early sample times and accounted for a mean of 2.3 % dose. Low amounts of two other radioactive components were detected, mainly at the later sample times (up to 96 hours post-dose). One component, designated HF-1, represented 1.0 % of the dose. The remaining component, designated HU-1, represented 1.9 % dose. The chemically identified urinary metabolites accounted for 98 % of the measured radioactivity in urine.

Analysis of faecal extracts by chromatography with radio-detection covered 89.5 % of the dose (93.5 % recovered radioactivity) and identified ANS9801-acid as the major component representing 52 % of the dose; component HF-1 represented 30 % of the dose.

Four-week and 12-week safety and tolerance studies of multiple daily doses of advantame were performed in 24 healthy subjects (12/sex) and in subjects with type II diabetes (36/sex/group), respectively (report ANSE-103a, 2006). Subjects were given 10 mg of advantame in 3 capsules/day corresponding to doses between 0.375 and 0.5 mg/kg bw. Despite a considerable inter and intra-individual variability, the results of these studies indicated that, following doses of 0.375 to 0.5 mg advantame/kg bw, systemic exposure to the parent substance is minimal. ANS9801-acid was the only product detected in plasma. According to the authors, these data confirmed a steady state with no evidence of accumulation of ANS9801-acid during the 4 and 12-week test phases in both healthy and type II diabetic subjects.

#### 3.1.2.3. Comparison of metabolite profiles in rats, dogs, and human

The applicant provided a comparison of the radioactive urinary and faecal metabolites of advantame, demonstrating similarities in the metabolism of advantame in the three species (AJO218, 2005). The three major metabolites identified in human urine ANS9801-acid, HF-1, and HU-1 were also present in the urine of rats and dogs. According to the applicant, ANS9801-acid and HF-1 were the major metabolites present in human faecal samples and they were also identified in faeces of dogs, based on chromatographic properties. ANS9801-acid was the only common compound present in human, dog

and rat faeces. The Panel noted that hydrolysis of the peptide bond within the ANS9801-acid molecule leads to the formation of both HF-1 and phenylalanine in all investigated models.

Despite the fact that low percentages of the administered radioactivity were observed in the blood, the Panel noted that this study (AJO218, 2005) did not compare the circulating metabolites observed in the plasma of rats, dogs and humans. In rats, the ANS9801-acid equivalents present in the plasma accounted for 6 to 11 % of the corresponding AUC value of the total plasma radioactivity whereas 89 to 94 % corresponded to unidentified circulating metabolites. In dogs, advantame and ANS9801-acid accounted for < 0.4 % of the plasma total radioactivity for all dose-routes and levels and > 99 % of the radioactivity was not identified. According to the applicant, sulphate conjugates of ANS9801-acid would contribute to the majority of ANS9801-acid equivalents of radioactivity in the dog (data not presented). Conversely in humans, ANS9801-acid represented 92.0 % of plasma radioactivity on comparison of AUC values. The Panel noted that only small amounts (8 %) of unidentified metabolites would be present in human plasma, by contrast to rats and dogs. This observation indicates the differences in metabolism of advantame in the three species. Upon request of the Panel, the applicant considered that all of the metabolites identified in human urine and faeces have been detected in either or both the urine and faeces of both rats and dogs. Since all of the plasma, urinary, and faecal metabolites detected in humans have been detected in rats and dogs, these animals constitute appropriate models from which to assess the safety of advantame. The Panel agreed with this conclusion.

The results based on the study reports provided in the application dossier on the pharmacokinetics and metabolism of advantame in the rat, dog, and man were published by Ubukata et al. (2011).

The results based on the study reports provided in the application dossier on the safety, tolerability and pharmacokinetic profile of advantame in healthy volunteers were published by Warrington et al. (2011).

### 3.1.3. Toxicokinetics of advantame derived from toxicity studies

The systemic exposure of various animal species to advantame and its metabolite ANS9801-acid was assessed as a part of different toxicity studies described in sections 3.2.2, 3.2.4 and 3.2.5: 13-week study in mice (AJO/174, 2002), 13-week study in rats (AJO/176, 2004), chronic toxicity and carcinogenicity study in rats (AJO/195, 2006), 1-year study in dogs (AJO/196, 2005) and a prenatal developmental toxicity study in rabbits (AJO/183, 2005). During these studies, blood samples were collected at various time intervals depending on the duration of each study. Advantame and its metabolite ANS9801-acid were measured by a validated LC-MS/MS method in plasma.

In each study, the rate of systemic exposure was expressed as the maximum mean plasma concentration ($C_{max}$) and the extent of systemic exposure as the area under the mean plasma concentration-time curve estimated up to 24 hours post-dose ($AUC_{24}$). These toxicokinetic parameters were derived for both advantame and ANS9801-acid.

Regarding results of the $C_{max}$, the Panel noted that in all studies, changes observed in this parameter ($C_{max}$) clearly correlate with those observed in the extent of systemic exposure expressed as $AUC_{24}$. Consequently, the Panel decided to present only the $AUC_{24}$ data set which is more representative of the internal dose distributed in animals. Moreover in all studies, $C_{max}$ of both advantame and ANS-9801 acid occurred in 0.5 to 1 hour post-dosing.

#### 3.1.3.1. Toxicokinetics of advantame in toxicity studies with dietary exposure of animals

***Subchronic toxicity study in mice***

As indicated in the 13-week study in mice (AJO/174, 2002), blood samples were taken during weeks 1, 6 and 12 of the study, following dietary administration of advantame at nominal dose levels of 5000, 15 000, and 50 000 mg/kg diet. The dietary concentrations of advantame resulted in doses of 0,

734, 2129, and 7444 mg/kg bw/day in males and 0, 892, 2593, and 9317 mg/kg bw/day in females. Plasma concentrations of advantame and ANS9801-acid were measured in samples taken at 6 time-points over a 24-hour period. The $AUC_{24}$ values are presented in Table 7.

**Table 7:** Areas under the mean plasma advantame and ANS9801-acid concentration-time curves over a 24-hour interval ($AUC_{24}$) during weeks 1, 6 and 12 of a 13-week study in mice

| Dose (mg advantame/ kg diet) | Dose (mg advantame/kg bw/day) | | $AUC_{24}$ **Advantame** (ngxh/ml) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | **Week 1** | | **Week 6** | | **Week 12** | |
| | **Males** | **Females** | **Males** | **Females** | **Males** | **Females** | **Males** | **Females** |
| 5000 | 734 | 892 | 1258 | 1824 | 751 | 849 | 1807 | 2294 |
| 15000 | 2129 | 2593 | 4149 | 4844 | 2340 | 3351 | 2517 | 9065 |
| 50000 | 7444 | 9317 | 7812 | 12431 | 3825 | 6196 | 6268 | 9125 |
| **Dose (mg advantame/ kg diet)** | **Dose (mg advantame/kg bw/day)** | | $AUC_{24}$ **ANS9801-acid** (ngxh/ml) | | | | | |
| | | | **Week 1** | | **Week 6** | | **Week 12** | |
| | **Males** | **Females** | **Males** | **Females** | **Males** | **Females** | **Males** | **Females** |
| 5000 | 734 | 892 | 12207 | 13811 | 8602 | 12704 | 6518 | 10953 |
| 15000 | 2129 | 2593 | 25591 | 42433 | 26624 | 46231 | 23133 | 36826 |
| 50000 | 7444 | 9317 | 90548 | 136701 | 95680 | 141734 | 97421 | 138790 |

The extent of systemic exposure of mice to advantame is highly variable and appeared to be characterised by non-linear (dose-dependent and time-dependent) kinetics and with no clear underlying mechanism over the nominal dose range 5000 to 50 000 mg/kg diet during weeks 1, 6 and 12. Increasing the dose of advantame above 5000 mg/kg diet is likely to result in a disproportionately lower systemic exposure to advantame than would be predicted from a linear relationship.

The rate and extent of systemic exposure of mice to ANS9801-acid appeared to be characterised by linear (dose-independent) kinetics over the nominal dose range 5000 to 50 000 mg/kg diet during weeks 1, 6 and 12.

***Subchronic toxicity study in rats***

As indicated in 13-week study in rats (AJO/176, 2004), blood samples were taken during weeks 1 and 13 of the study, following dietary administration of advantame at nominal dose levels of 1500, 5000, 15 000 and 50 000 mg/kg diet. The dietary concentrations of advantame resulted in achieved doses of 0, 118, 415, 1231, and 4227 mg/kg bw/day in males and 0, 146, 481, 1487, and 5109 mg/kg bw/day in females. Plasma concentrations of advantame and ANS9801-acid were measured in samples taken at 6 time-points over a 24-hour period. The $AUC_{24}$ values are presented in Table 8.

**Table 8:** Areas under the mean plasma advantame and ANS9801-acid concentration-time curves over a 24-hour interval ($AUC_{24}$) during weeks 1 and 13 of a 13-week study in rats

| Dose (mg advantame/ kg diet) | Dose (mg advantame/kg bw/day) | | $AUC_{24}$ **Advantame** (ngxh/ml) | | | |
|---|---|---|---|---|---|---|
| | | | Week 1 | | Week 13 | |
| | Males | Females | Males | Females | Males | Females |
| 1500 | 118 | 146 | 644 | 1117 | 720 | 1051 |
| 5000 | 415 | 481 | 669 | 1003 | 620 | 1163 |
| 15000 | 1231 | 1487 | 3440 | 2653 | 5675 | 8680 |
| 50000 | 4227 | 5109 | 4222 | 6754 | 8312 | 4091 |
| **Dose (mg advantame/ kg diet)** | **Dose (mg advantame/kg bw/day)** | | **$AUC_{24}$ ANS9801-acid** (ngxh/ml) | | | |
| | | | Week 1 | | Week 13 | |
| | Males | Females | Males | Females | Males | Females |
| 1500 | 118 | 146 | 2654 | 2183 | 2510 | 2015 |
| 5000 | 415 | 481 | 5548 | 4820 | 3095 | 4483 |
| 15000 | 1231 | 1487 | 20271 | 25994 | 10427 | 10325 |
| 50000 | 4227 | 5109 | 52603 | 46427 | 26027 | 25687 |

The extent of systemic exposure of rats to advantame and the metabolite ANS9801-acid, is highly variable and appeared to be characterised by non-linear (dose-dependent and time-dependent) kinetics and with no clear underlying mechanism over the nominal dose range 1500 to 50 000 mg/kg diet during weeks 1 and 13. Increasing the dose of advantame above 1500 mg/kg diet is likely to result in a disproportionately lower systemic exposure to advantame and ANS9801-acid than would be predicted from a linear relationship.

***Chronic toxicity study in rats***

As indicated in the chronic toxicity and carcinogenicity study in rats (AJO/195, 2006), blood samples were taken during weeks 14, 26, 52 and 104 of the study, following dietary administration of advantame at nominal dose levels of 2000, 10 000 and 50 000 mg/kg diet. The dietary concentrations of advantame resulted in achieved doses of 0, 97, 488, and 2621 mg/kg bw/day in males and 0, 125, 630, and 3454 mg/kg bw/day in females. Plasma concentrations of advantame and ANS9801-acid were measured in samples taken at 6 time-points over a 24-hour period. The $AUC_{24}$ values are presented in Table 9.

**Table 9:** Areas under the mean plasma advantame and ANS9801-acid concentration-time curves over a 24-hour interval ($AUC_{24}$) during weeks 14, 26, 52 and 104 of a chronic toxicity and carcinogenicity study in rats

| Dose (mg advantame/ kg diet) | Dose (mg advantame/kg bw/day) | | $AUC_{24}$ **Advantame** (ngxh/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Week 14 | | Week 26 | | Week 52 | | Week 104 | |
| | Males | Females | Males | Females | Males | Females | Males | Females | Males | Females |
| 2000 | 97 | 125 | 595 | 813 | 1020 | 1560 | 661 | 509 | 426 | 490 |
| 10000 | 488 | 630 | 393 | 840 | 1880 | 1630 | 731 | 1180 | 1140 | 981 |
| 50000 | 2627 | 3454 | 1660 | 1720 | 1520 | 1730 | 935 | 1900 | 1510 | 3490 |
| **Dose (mg advantame/ kg diet)** | **Dose (mg advantame/kg bw/day)** | | **$AUC_{24}$ ANS9801-acid** (ngxh/ml) | | | | | | | |
| | | | Week 14 | | Week 26 | | Week 52 | | Week 104 | |
| | Males | Females | Males | Females | Males | Females | Males | Females | Males | Females |
| 2000 | 97 | 125 | 142 | 245 | 128 | 216 | 84.4 | 89.8 | 130 | 64.2 |
| 10000 | 488 | 630 | 537 | 378 | 661 | 658 | 448 | 468 | 321 | 197 |
| 50000 | 2627 | 3454 | 1710 | 2910 | 2500 | 4450 | 959 | 3310 | 513 | 1880 |

The extent of systemic exposure of rats to advantame is highly variable and appeared to be characterised by non-linear (dose-dependent and time-dependent) kinetics and with no clear underlying mechanism over the nominal dose range 2000 to 50 000 mg/kg diet during weeks 14, 26, 52 and 104 of the study. Increasing the dose of advantame above 2000 mg/kg diet is likely to result in a lower systemic exposure to advantame than would be predicted from a linear relationship.

The extent of systemic exposure of male rats to the metabolite ANS9801-acid appeared to be characterised by non-linear (dose-dependent) kinetics over the nominal dose range 2000 to 50 000 mg/kg diet during the study. However, in females the kinetics appeared to be non-linear (dose-dependent) during week 14 but dose-independent (linear) during weeks 26, 52 and 104. Increasing the dose of advantame to male rats above 10 000 mg/kg diet is likely to result in a lower systemic exposure to ANS9801-acid than would be predicted from a linear relationship.

According to the applicant, despite the few differences in dose proportionality between male and female rats, overall, there would not be any consistent sex-related differences in the $C_{max}$ and $AUC_{24}$ values of advantame or ANS9801-acid and the systemic exposure generally appeared to be similar during weeks 14, 26, 52 and 104 of the study.

***Subchronic toxicity study in dogs***

As indicated in a 1-year study in dogs (AJO/196, 2005), blood samples were taken on day 1 and during weeks 13, 27 and 52 of the study, following daily dietary administration of advantame at nominal dietary concentrations of 2000, 10 000 and 50 000 mg/kg diet. Plasma concentrations of advantame and ANS9801-acid were measured in samples taken up to 24 hours after presentation of treated diet. The dietary concentrations of advantame resulted in achieved doses of 0, 82.5, 420.9, and 2058 mg/kg bw/day in males and 0, 81.9, 406.2, and 2139 mg/kg bw/day in females. The $AUC_{24}$ values are presented in Table 10.

**Table 10:** Areas under the mean plasma advantame and ANS9801-acid concentration-time curves over a 24-hour interval ($AUC_{24}$) during on day 1 and during weeks 13, 27 and 52 of a 1-year study in dogs

| Dose (mg advantame/ kg diet) | Dose (mg advantame/kg bw/day) | | **$AUC_{24}$ Advantame** (ngxh/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Day 1 | | Week 13 | | Week 27 | | Week 52 | |
| | Males | Females | Males | Females | Males | Females | Males | Females | Males | Females |
| 2000 | 82.5 | 81.9 | 23.5 | 31.3 | 24.2 | 28.3 | 12.8 | 16.1 | 6.93 | 7.01 |
| 10000 | 420.9 | 406.2 | 170 | 145 | 73.4 | 61.2 | 47.9 | 70.9 | 37.8 | 37.3 |
| 50000 | 2058 | 2139 | 581 | 765 | 353 | 755 | 233 | 730 | 148 | 388 |
| **Dose (mg advantame/ kg diet)** | **Dose (mg advantame/kg bw/day)** | | **$AUC_{24}$ ANS9801-acid** (ngxh/ml) | | | | | | | |
| | | | Day 1 | | Week 13 | | Week 27 | | Week 52 | |
| | Males | Females | Males | Females | Males | Females | Males | Females | Males | Females |
| 2000 | 82.5 | 81.9 | 5350 | 8010 | 8750 | 10100 | 6440 | 4470 | 4470 | 8240 |
| 10000 | 420.9 | 406.2 | 28400 | 30300 | 22300 | 22000 | 15600 | 17900 | 17900 | 13700 |
| 50000 | 2058 | 2139 | 42100 | 51400 | 46900 | 130000 | 27000 | 27500 | 27500 | 39500 |

The rate and extent of systemic exposure of dogs to advantame on day 1 and during weeks 13, 27 and 52 of the study appeared to be characterised by dose-independent (linear) kinetics over the dietary concentration range 2000 to 50 000 mg/kg diet. The rate and extent of systemic exposure of male and female dogs to the metabolite, ANS9801-acid, was characterised by non-linear (dose-dependent) kinetics over the dietary concentration range 2000 to 50 000 mg/kg diet throughout the study. Increasing the dietary concentration of advantame above 2000 mg/kg diet, it is likely to result in a disproportionately lower systemic exposure to ANS9801-acid than would be predicted from a linear relationship.

In addition, the study also provided evidence that the systemic exposure of female dogs to advantame and ANS9801-acid was generally higher than that of males, and after repeated administration of

advantame the rate and extent of systemic exposure of dogs to advantame and ANS9801-acid were generally lower than those values after a single dose, but exposure of dogs to ANS9801-acid were higher at the lowest dietary concentration.

3.1.3.2. Toxicokinetics of advantame from a prenatal developmental toxicity study in rabbits

As indicated in the prenatal developmental toxicity study in rabbits (AJO/183, 2005), blood samples were taken on days 6 and 27 of pregnancy (days 1 and 22 of treatment) of the study, in order to assess the systemic exposure of pregnant female rabbits to advantame and its metabolite ANS9801-acid, following daily oral gavage administration of advantame at dose levels of 500, 1000 and 2000 mg/kg bw/day from day 6 to 27 after mating. Plasma concentrations of advantame and ANS9801-acid (both measured as anhydrous bases) were measured in samples taken up to 24 hours post-dose. The $AUC_{24}$ values are presented in table 11.

**Table 11:** Areas under the mean plasma advantame or ANS9801-acid metabolite concentration-time curves over a 24-hour interval ($AUC_{24}$) on days 6 and 27 of gestation (days 1 and 22 of treatment) during a prenatal developmental toxicity study in rabbits

| **Dose** (mg advantame/kg bw/day) | **Advantame** | | **ANS9801-acid** | |
|---|---|---|---|---|
| | **$AUC_{24}$** (ngxh/ml) | | **$AUC_{24}$** (ngxh/ml) | |
| | **Day 6** | **Day 27** | **Day 6** | **Day 27** |
| 500 | 4982 | 3906 | 151896 | 147414 |
| 1000 | 3737 | 11594 | 117112 | 268999 |
| 2000 | 8382 | 22367 | 274417 | 486206 |

The rate and extent of systemic exposure of pregnant female rabbits to advantame is highly variable and appeared to be characterised by non-linear (dose-dependent and time-dependent) kinetics and with no clear underlying mechanism over the dose range 500 to 2000 mg/kg bw/day on day 6 after mating during the oral prenatal developmental toxicity study. Increasing the dose of advantame above 500 mg/kg bw/day is likely to result in a disproportionately lower systemic exposure to advantame than would be predicted from a linear relationship.

At the two higher dose levels (1000 and 2000 mg/kg bw/day) the $AUC_{24}$ values were higher after repeated oral doses (day 27) than those values after a single dose (day 6), and these differences were statistically significant ($p < 0.001$). The accumulation ratios ($AUC_{24}$ at day 27/$AUC_{24}$ at day 6) were greater than one at the 1000 and 2000 mg/kg bw/day dose levels, indicating that accumulation occurred after repeated oral administration of advantame at these dose levels. At this stage (day 27), there was some evidences that increasing the dose of advantame resulted in a disproportionately higher systemic exposure to advantame than would be predicted from a linear relationship, however, the extent of this disproportionality was not great.

The rate and extent of systemic exposure of pregnant female rabbits to ANS9801-acid, appeared to be characterised by non-linear (dose-dependent) kinetics over the dose range 500 to 2000 mg advantame/kg bw/day on day 6 and day 27 after mating. Increasing the dose of advantame above 500 mg/kg bw/day is likely to result in a disproportionately lower systemic exposure to ANS9801-acid than would be predicted from a linear relationship. As for advantame, at the two higher dose levels (1000 and 2000 mg/kg bw/day) the $AUC_{24}$ values were higher after repeated oral doses (day 27) than those values after a single dose (day 6), and these differences were statistically significant ($p < 0.001$). The accumulation ratios were greater than one at the 1000 and 2000 mg/kg bw/day dose levels. According to the applicant accumulation of ANS9801-acid occurred after repeated oral administration of advantame at these dose levels.

### 3.1.4. Summary of the ADME data

The Panel noted that the terminology in each of the individual study reports provided in the application dossier was not consistent; therefore further clarification and overall evaluation of the

metabolism and of the pharmacokinetics data was requested from the applicant. The applicant provided supplementary information[8] and a publication in the scientific literature (Ubukata et al., 2011) summarising the *in vivo* studies on the metabolism of advantame was also brought to the Panel's attention. Using the information from the available study reports, the supplementary information and the published Ubukata et al. (2011) study, the Panel has summarised the available information.

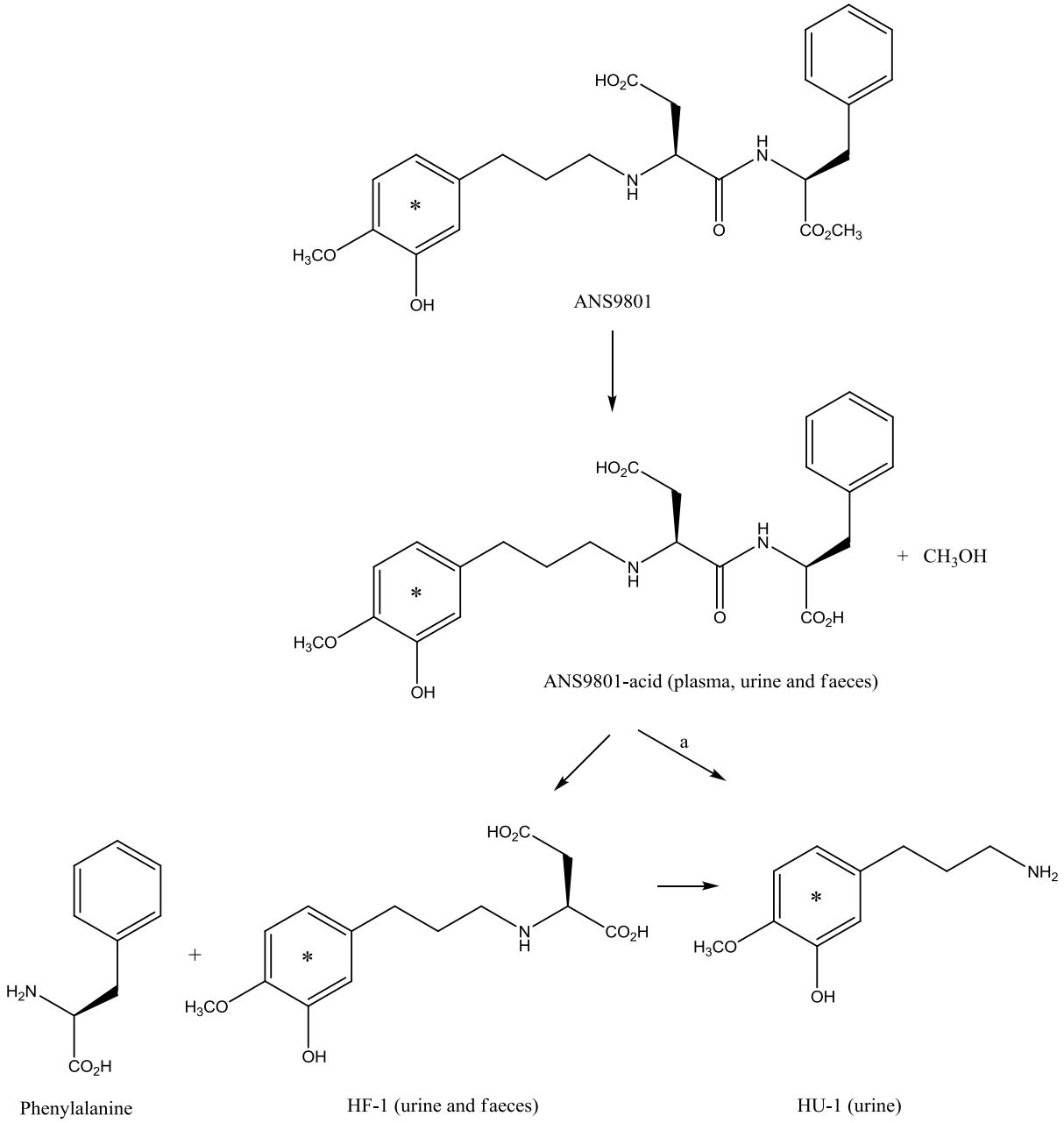
Overall, ADME studies in rats and dogs indicated that radioactivity from $^{14}$C-advantame is rapidly absorbed, but only to a limited extent, with oral bioavailability between 7 and 13 %. In both species, advantame was rapidly converted in the gastrointestinal tract and plasma to ANS9801-acid. In these animal species, a proportion of plasma radioactivity could not be chemically identified. Ubukata et al. (2011) suggested that the unidentified plasma radioactivity could be one or more ANS9801-acid conjugates. Based on all the available data, the Panel considered this explanation would be plausible. Absorption appeared to be prolonged in the dog because of the presence of a circulating sulphate conjugate in this animal species. Based on the data from the *in vitro* stability studies with simulated gastric and intestinal fluids, the majority of advantame conversion appeared to occur in the gastrointestinal tract prior to absorption.

Tissue radioactivity was relatively low and restricted to liver and kidneys. Following oral dosing, radioactivity from advantame was found in the faeces (82-89 %), with urinary excretion representing a minor route (2-7 %). ANS9801-acid was the predominant metabolite found in urine and faeces of both species, while only minor amounts of free ANS9801-acid were found in plasma. This metabolite was hydrolysed to form the aspartic acid derivative HF-1 which was found in the faeces of dogs, but not in rats. For dogs three metabolites were identified in urine, ANS9801-acid (3 % of the administered dose), HU-1 (0.3 % of the administered dose) and HF-1 (1.3 % of the administered dose). There was also 1 % of the administered dose that was not identified. In urine of rats, ANS9801-acid (0.3 % of the administered dose), HU-1 (0.3 % of the administrated dose) and HF-1 (0.3 % of the administrated dose) were also identified whereas 0.7 % of the dose was unidentified metabolite. In addition to ANS9801-acid, other minor metabolites were isolated from the faeces of rats, including RF-1 and another unidentified metabolite (RF-2).

After an oral dose, advantame radioactivity was rapidly but poorly absorbed in humans. Although metabolism of advantame to ANS9801-acid occurred rapidly, only trace amounts of advantame were detected in plasma and none in excreta. In human, the proportion of plasma radioactivity chemically identified was higher and was the ANS9801-acid. However, the excretion of radioactivity was predominantly recovered in faeces (89 % of the dose) and urine (6.2 % of the dose). In faecal extracts the identified metabolites were ANS9801-acid and HF-1, 52 % and 30 % of the dose, respectively. In urine, three metabolites were identified, ANS9801-acid (2.3 % of the dose), HU-1 (1.9 % of the dose) and HF-1 (1 % of the dose). The Panel noted that the hydrolysis of the peptide bond within the ANS9801-acid molecule leading to the formation of either HU-1 or HF-1 also releases phenylalanine. An additional chronic study confirmed a steady state in plasma with no evidence of accumulation of ANS9801-acid during the 4 and 12-week test phases in healthy and type II diabetes subjects.

The metabolic fate of $^{14}$C-advantame in humans is summarised in Figure 3. This is based on the preclinical studies in animals and clinical studies in humans using radioactive $^{14}$C-advantame labelled in the phenol group.

[8] Additional information submitted to EFSA on 9th November 2011.




* Denotes position of radiolabel
[a] As hypothetised by Ubukata et al. (2011) in light of lacking information on the fate of unlabelled portion of the molecule

**Figure 3:** Metabolic pathways of advantame based on pre-clinical and clinical studies in humans.

Toxicokinetic parameters describing the systemic exposure of various animal species to advantame and its metabolite ANS9801-acid were assessed as a part of different toxicity studies: 13-week study in mice (AJO/174, 2002), 13-week study in rats (AJO/176, 2004), chronic toxicity and carcinogenicity study in rats (AJO/195, 2006), 1-year study in dogs (AJO/196, 2005) and a prenatal developmental toxicity study in rabbits (AJO/183, 2005).

Toxicokinetic data derived from dietary subchronic or chronic toxicity studies in dietary administered mice, rats and dogs, demonstrated that in all animal species the $C_{max}$ of advantame and ANS9801-acid were observed 0.5 to 1 hour post-dosing, indicating a rapid absorption and metabolism of advantame.

In all studies, whatever the dose was, the $AUC_{24}$ of ANS9801-acid were 5 to 300-fold higher than those of advantame depending on the animal species, indicating an extensive metabolism of advantame to its metabolite, ANS9801-acid. In the same studies, the rate and extent of systemic exposure of animals to advantame and ANS9801-acid were highly variable and appeared to be characterised by non-linear kinetics and with no clear underlying mechanism over the dietary concentration range used in these experiments. By considering the changes in $AUC_{24}$ data in the course of these studies, the Panel considered that advantame or ANS9801-acid do not accumulate even in the case of high dietary doses given to these animal species for periods as long as 52 or 104 weeks.

The Panel noted that pharmacokinetic data derived from a prenatal developmental toxicity study in rabbits demonstrated that when advantame administered by gavage at a similar dose of around 2000 mg advantame/kg bw/day, the systemic exposure to both advantame and ANS9801-acid is 5 to 25 times higher than in dietary administered animals. This would represent a worst case situation for a maximal systemic exposure of animals to advantame and ANS9801-acid. The Panel also noted that in this study, advantame and ANS9801-acid are described to accumulate at the higher doses since increasing the dose of advantame resulted in a disproportionately higher systemic exposure to advantame than would be predicted from a linear relationship.

Overall, the Panel considered that the high variability and the lack of clear underlying mechanism did not permit such a clear conclusion on accumulation or non-linearity in kinetics.

### 3.2. Toxicological data

The Panel noted that the studies provided by the applicant were performed according to the OECD guidelines for testing of chemicals and the good laboratory practice (GLP).

#### 3.2.1. Acute oral toxicity

An acute oral toxicity study was performed in Han Wistar-derived rats (5/sex/group) (report AJO/155, 2001). A single dose of 5000 mg advantame/kg bw (100.1 % purity) was administered orally by gavage in a 1 % w/v aqueous methylcellulose solution (20 ml/kg bw). The animals were observed for 15 days. There was no mortality at any time during the study. The only clinical signs related to treatment were the presence of white coloured faeces in all the rats. No macroscopic changes were found at necropsy. The acute oral $LD_{50}$ for advantame in rats was > 5000 mg/kg bw.

#### 3.2.2. Subacute and subchronic toxicity

Subchronic toxicity studies with advantame in mice, rats, and dogs were submitted by the applicant.

According to the applicant, a 13-week study in the mouse was designed as a dose-range finding study for a 2-year carcinogenicity study and to assess the systemic toxic potential of advantame (report AJO/174, 2002). In consequence haematology, blood chemistry, urinalysis, and ophthalmologic examinations were not performed. Advantame (99.5 %) was administered at concentrations of 0, 5000, 15 000, or 50 000 mg/kg diet to Charles River CD-1 mice (20/sex/group) for a period of 13 weeks. The dietary concentrations of advantame resulted in achieved doses of 0, 734, 2129, and 7444 mg/kg bw/day in males and 0, 892, 2593, and 9317 mg/kg bw/day in females. All animals apart from one survived to the end of the study: one male treated at 15 000 mg/kg/diet was euthanized in week 11 due to severe superficial abrasions, which were not treatment-related. The clinical appearance of mice in the treated groups was similar to that of controls. There were no statistically significant differences in body weight. The body weight gain for males was 15.7, 15.0, 15.5, 14 g and 9.0, 8.1, 9.4, 8.0 g for females for the control, low, mid and high-dose groups, respectively, and was not statistically significantly different from the controls for both sexes Food consumption was similar in all groups at all times except for higher figures for the male high-dose group at weeks 1 ($p < 0.05$) and 2 ($p < 0.05$) and the female high-dose group in week 3 ($p < 0.01$). Food conversion efficiency was similar in all groups apart from lower values for high dose males in weeks 4 and 5 ($p < 0.01$) and high dose females in week 3 ($p < 0.05$). Absolute and relative organ weights of the treated groups were not statistically significantly different from those of control groups. Macroscopic examination did not reveal any

differences between the treated and the control groups. No histological examination was performed on any of the organs and tissues. According to the Panel, the limited data collected indicated a NOAEL of 50 000 mg advantame/kg diet, the highest dietary concentration tested, equal to 7444 mg/kg bw/day for males and 9317 mg/kg bw/day for females. The Panel noted the limited design of this study.

In a 4-week preliminary toxicity study, advantame (99.2 % purity) was administered at concentrations of 0, 1500, 15 000, 30 000, or 50 000 mg/kg diet to Han Wistar rats (10/sex/group) for a period of 28 (males) or 29 (females) days (report AJO/151, 2002). The dietary concentrations of advantame resulted in achieved doses of 0, 174, 1739, 3672, and 6126 mg/kg bw/day in males and 0, 187, 1920, 3739, and 6490 mg/kg bw/day in females. All animals survived to the end of the study. There was no difference in clinical appearance of treated animals compared to the controls. In the high-dose animals of both sexes, body weight throughout the study and body weight gain (82 to 85 % of controls for males and females respectively) were statistically significantly lower compared to the controls. There was no clear effect of treatment on food consumption in any of the treatment groups but food conversion efficiency for the high-dose group was lower compared to the controls (81 % and 86 % of controls for males and females respectively). Water intake in week 1 was statistically significantly higher than that of controls in male groups treated with 15 000 mg/kg diet or above and in female groups treated with 30 000 mg/kg diet or above. In week 3 water intake was statistically significantly higher in both sexes at the highest dose (129 % and 110 % of the control). Several haematological parameters in the treated groups showed statistically significant difference from the controls. For males, there were lower hematocrit and haemoglobin concentrations in all treated groups and lower mean cell haemoglobin, monocyte counts and prothrombin time in the 50 000 mg/kg diet group. For females low white blood cell and lymphocyte counts were found in all treatment groups and a lower count of basophils and of large unstained cells at the 50 000 mg/kg diet group. Statistically significant differences in clinical chemistry parameters between the treated groups and the controls included for males decreased alanine aminotrasferase (ALAT) at 30 000 and 50 000 mg/kg diet, increased urea at and above 15 000 mg/kg diet and slightly low chloride and beta globulin at 30 000 and 50 000 mg/kg diet. For females decreased ALAT and alkaline phosphatase (ALKP) and slightly higher sodium at 50 000 mg/kg diet were reported. The Panel noted the lack of a consistent dose-response relationship and sex-related inconsistencies in the altered haematological and clinical chemistry parameters.

In the results of urinalysis a statistically significantly higher specific gravity was found for females at the two highest doses and it was coincident with a non-significant reduction in urine volume. Absolute and relative organ weights in all treated female groups and of male groups treated up to 30 000 mg/kg diet were similar to those of controls with the exception of the absolute heart weight of males in the 30 000 mg/kg diet group (0.972 g control vs. 0.892 g; $p < 0.05$). In the male high-dose group absolute weights of brain, heart, kidneys, lungs and bronchi, testes and thymus were statistically significantly different from those of controls but the difference was eliminated when weights were expressed relative to body weight except for relative thymus weight (0.1932 control vs. 0.1582, $p < 0.01$). This finding was not associated with any histopathological changes in the organ. Histopathological examination revealed increased incidence of cortico-medullary mineralization in the kidneys of females receiving 1500 mg/kg diet or above. The incidence increased with dose (2/10, 3/10, 4/10, 7/10, 8/10; trend analysis $p< 0.01$) and the incidence at the highest dose was significantly different ($p < 0.05$) from controls. The Panel considered this finding to be treatment-related but not of toxicological significance as it is frequently found in rats given diets that modify electrolyte homeostasis, such as those containing high levels of non-nutritive substances. The Panel also noted that this finding was not seen after 13 weeks of treatment in the rat subchronic study reported below. According to the Panel the NOAEL was 30 000 mg advantame/kg diet equal to 3672 mg/kg bw/day in males and 3739 mg/kg bw day in females based on effects on body weight gain.

Based on the results of the 4-week study, advantame (99.5 % purity) was administered in the diet for 13 weeks at concentrations of 0, 1500, 5000, 15 000, or 50 000 mg/kg to groups of 20 male and 20 female HsdBrl Han: Wist (Han Wistar) rats (report AJO/176, 2004). An additional 5 rats/sex/group 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. The dietary concentrations of advantame resulted in achieved

doses of 0, 118, 415, 1231, and 4227 mg/kg bw/day in males and 0, 146, 481, 1487, and 5109 mg/kg bw/day in females. No treatment-related deaths occurred. The clinical appearance of treated and control animals was similar and there were no statistically significant differences in body weight, body weight gain, food consumption, or food conversion efficiency in the treated groups compared to the control group. Water intake was statistically significantly higher than for controls for both sexes at the highest dose and for females from the 15 000 mg/kg diet group in weeks 1, 5, and 11. Additionally in week 11 a statistically increased water intake was recorded for males from the 5000 and 15 000 mg/kg diet groups. During week 3 of the recovery period the water consumption for both sexes that previously received 15 000 or 50 000 mg/kg diet was similar to or not statistically significantly lower than control values. No evidence of neurotoxicity was observed in the results of the functional observation battery (FOB) testing performed throughout the treatment period and during week 3 of the recovery period. No changes attributable to treatment were reported for ophthalmoscopic examination.

The haematological analyses revealed some statistically significant differences to the controls. Low haemoglobin and haematocrit at 50 000 mg/kg diet, decreased counts of leucocyte, lymphocyte and of large unstained cells at 15 000 and 50 000 mg/kg diet, and reduced activated partial thromboplastin time at 50 000 mg/kg diet were reported for males. Low haemoglobin and haematocrit at 15 000 and 50 000 mg/kg diet, decreased count of erythrocytes, lymphocytes, monocytes and of large unstained cells, and reduced prothrombin time at 50 000 mg/kg diet were reported for females. The Panel noted that at the end of the recovery period there were no statistically significant differences in these parameters between treated and control animals. The blood chemistry analyses revealed some statistically significant differences from the controls. For males these included decreased ALAT at 50 000 mg/kg diet, lower bilirubin from and above 1500 mg/kg diet, increased glucose at 5000 and 15 000 mg/kg diet, lower sodium at 15 000 mg/kg diet, lower potassium and calcium from and above 1500 mg/kg diet, lower phosphorus from and above 5000 mg/kg diet, lower total protein, albumin and albumin to globulin ratio from and above 1500 mg/kg diet. The Panel noted that for calcium and phosphorus differences from the control remained following 4-week recovery period. For females the differences from the controls included: decreased ALAT and ornithine-carbamyl transferase from and above 15 000 mg/kg diet, increased aspartate aminotransferase at 1500 mg/kg diet, lower bilirubin from and above 5000 mg/kg diet, increased urea from and above 15 000 mg/kg diet, increased creatinine at 50 000 mg/kg diet, decreased glucose at 50 000 mg/kg diet, increased phospholipids at 1500 mg/kg diet, increased sodium, decreased potassium and chloride at 50 000 mg/kg diet, decreased calcium from and above 15 000 mg/kg diet, and lower albumin to globulin ratio at 50 000 mg/kg diet. The Panel noted lack of an apparent dose-response relationship for changes in blood chemistry parameters. The following urinalysis parameters were statistically significantly different from the controls in males: a higher specific gravity at 15 000 mg/kg diet, increased sodium at 50 000 mg/kg diet, increased potassium at 1500 and 5000 mg/kg diet. For females the reported differences in urinalysis included a decreased urine volume at and above 1500 mg/kg diet, decreased sodium, potassium and chloride from and above 5000 mg/kg diet. The Panel noted that the lower values for sodium, potassium and chloride in females remained after the recovery period but the differences from the controls were no longer statistically significant. The Panel further noted that none of the findings followed a dose-response relationship.

The results of the immunotoxicity assays showed a slight but statistically significant reduction in lymphocytes mitogenic responsiveness to Concanavalin A in treated males at all doses after 4 weeks but not after 13 weeks of treatment or at the end of the 4-week recovery period. Several parameters indicative of potential immunotoxicity showed statistically significant differences from the controls. For males there was a reduction of total T cells at all doses in week 4 and at 50 000 mg/kg diet in week 13; there was also an increase in natural killer (NK) cells (CD3-NKRP1)(NKR cells) at 50 000 mg/kg diet in week 13. For females at week 4 there was a decrease in total B cells at 5000 mg/kg and all doses above; at week 13 there was an increase of total T cells at 15 000 and 50 000 mg/kg diet, and a decrease in NK cells at 5000 mg/kg diet. The Panel noted no obvious dose-response relationship for the statistically significant changes in the percentage of certain lymphocyte subpopulations and their occurrence in single sexes. No statistically significant effects on relative

thymus weight were reported after 13 weeks of treatment nor were there statistically significant differences in relative weights of other organs as compared to the controls.

Macroscopic examination of animals killed on completion of the treatment or recovery periods revealed no significant findings. There was a high incidence of pale faeces in the gastro-intestinal tract of animals given 50 000 mg/kg diet which was not present at the end of the recovery period. There were no macroscopic or histopathological findings attributed to treatment with advantame. The incidence of interstitial inflammatory infiltration in the prostate in treated rats which was up to 20 % (control: 0/20, 1500 mg/kg diet: 3/20, 5000 mg/kg diet: 2/20, 15 000 mg/kg diet: 0/19, 50 000 mg/kg diet: 4/20), or 11 % (9/80), when all treated males were considered was not higher than that observed in recovery controls (1/5; 20 %). The Panel noted that the inflammatory infiltration of the prostate is a relatively common background pathology finding in rats and therefore not likely to be related to treatment. A slight increase (not statistically significant) in the incidence of medullary mineralisation of the kidneys after 13 weeks in females (8/20, 9/20, 8/20, 7/20 and 13/20, at 0, 1500, 5000, 15 000 and 50 000 mg/kg diet, respectively) was considered by the Panel to be a random finding, since this is a relatively common background finding in rats of this age, and the severity was reported as minimal in majority of the animals. There were no apparent treatment-related differences in the incidence of this finding in animals killed at the end of the recovery period. An increase in the incidence of degeneration of optic nerve fibres after 13 weeks was reported in females (3/20, 4/20, 5/20, 7/20, 11/20, at 0, 1500, 5000, 15 000 and 50 000 mg/kg diet, respectively) which reached statistical significance at the highest dose ($p < 0.01$). These were unilateral findings and occasionally associated with unilateral degenerative changes in the harderian glands. The authors of the study considered that these changes were related to the blood sampling procedure and not to treatment with advantame. The Panel considered it a plausible explanation as damage to the eye and surrounding tissue may occur with use of this blood sampling technique. The Panel noted that several haematological and immunotoxicity end-points in groups receiving 15 000 or 50 000 mg advantame/kg diet were statistically significantly different from the controls after 13 weeks of treatment and some blood chemistry parameters showed statistically significant differences from the control at lower doses. The Panel noted that a dose-response was not always apparent, that there was no consistency between the sexes in the recorded changes and that they were not accompanied by morphological changes in any organs. Several of these differences were recorded for parameters recognised as variable in the laboratory rat. These changes if present as isolated findings could be regarded as random findings. However, the relation to treatment was suggested for several of these changes by the absence of significant differences in the end of the recovery period. The Panel further noted that a decreased number of WBC and lymphocytes was also seen in the 4-week rat study.

Male and female beagle dogs (4/sex/group) were given diets containing advantame (99.2 % purity) at concentrations of 0, 5000, 15 000, or 50 000 mg/kg for a period of 4 weeks (report AJO/156, 2002). The dietary concentrations of advantame resulted in achieved doses of 0, 232, 737, and 2385 mg/kg bw/day in males and 0, 254, 743, and 2488 mg/kg bw/day in females. There were no deaths during the study. Pale faeces were observed in the high-dose animals. Male and female dogs receiving 50 000 mg/kg diet gained less weight than other groups during the treatment period (males: 1.2, 1.3, 0.8, 0.7 kg; females: 1.2, 0.9, 1.1, 0.7 kg (58 % of the control value; $p < 0.05$) in the control, low, middle and high-dose groups respectively). The lower body weight gain was considered by the authors of the study mainly due to outlier data from one dog of each sex, which gained only 0.1 kg (male) and 0.3 kg (female) respectively during the period. The authors of the study considered the lower body weight gain in the female high-dose group to be due to normal animal variation and therefore of doubtful toxicological importance. Most animals were eating maximal or near maximal amounts of food before treatment and this continued during the treatment period. Food consumption was not statistically significantly different in the treated groups compared to the control group. Increased mean cell haemoglobin concentration and lymphocyte counts were seen in males at the highest dose and increased lymphocyte counts were also seen in the male group receiving 15 000 mg/kg diet. Increased urea was found in males of the 15 000 mg/kg and 50 000 mg/kg diet groups; decreased glucose values were seen in all male treated groups. For females increased sodium was found in the two highest dose groups and decreased chloride in the 50 000 mg/kg diet group. The Panel noted that these differences

occurred in a non-dose-dependent manner, were limited to one sex, and were within physiological ranges (Evans, 2009a,b). Urinalysis revealed no differences between treated and control groups. Small but statistically significant changes were found in the electrocardiography (ECG). At 24 hours post-dose during week 2 the group mean values relating to the heart rate were increased in females at the two highest doses. The authors reported that this trend was seen in the pre-dose period. In the absence of a strict trend with increasing dosage the finding was not considered by the authors to be related to treatment. During week 4 at 1 hour post-dose the group mean PR interval in males at the highest dose was slightly but statistically significantly increased compared to the controls. The authors of the study reported that the value was within the range of historical control data and therefore considered that this finding was not related to treatment.

The organ weights of all treated dogs of both sexes were similar to those of the controls apart from absolute thymus weights for both sexes of all of the treated groups which were lower than those of controls. The difference achieved statistical significance for the adjusted means of males receiving 15 000 mg/kg diet or 50 000 mg/kg diet and for absolute (unadjusted) weights for females from all treated groups, but the microscopic picture of the thymus was not altered. In addition, there was a minor but statistically significant increase (12 %) in absolute (unadjusted) brain weight for males receiving 50 000 mg/kg diet compared with the control. This finding was not accompanied by histopathological changes and was limited to one sex. There were no macroscopic or microscopic pathology findings attributable to advantame treatment in any of the organs or tissues examined. The Panel considered that the reduced body weight gain in the high-dose group could not be explained by a difference in feed intake and it is therefore an adverse effect of treatment. All other differences occurred either in the high dose group alone or are considered by the Panel to be unrelated to treatment. According to the Panel the NOAEL was 15 000 mg advantame/kg diet, equal to 737 mg/kg bw/day in males and 743 mg/kg bw/day in females.

In a 13-week study in beagle dogs (4/sex/group) advantame (99.5 % purity) was administered at concentrations of 0, 5000, 15 000, or 50 000 mg/kg diet (report AJO/179, 2005). The dietary concentrations of advantame resulted in achieved doses of 0, 205, 667, and 2230 mg/kg bw/day in males and 0, 229, 703, and 2416 mg/kg bw/day in females. All animals survived to the end of the study. Clinical appearance of the treated and control animals was similar for all groups although loose and/or liquid faeces were observed in all treated animals, the severity of which appeared to increase with dose, and the faeces in the high-dose group of both sexes were pale. The body weight gain of high-dose males was statistically significantly reduced (30 % of control) but the body weight gains for all other treated groups were comparable to the concurrent controls. Food consumption and results of ophthalmoscopic and ECG examinations were similar for all groups. Statistically significantly lower haematocrit, haemoglobin concentration and erythrocyte counts were seen at week 13 in females from the groups receiving 15 000 or 50 000 mg/kg diet. Females at the highest dose also had lower reticulocyte and leukocyte counts. The Panel noted that the haemoglobin concentration, reticulocyte and leukocyte counts were also statistically significantly lower in the high-dose group in the pre-treatment period, but that all values were within the physiological range for the species (Evans, 2009a), and within the range of historical controls. Blood chemistry examination in week 6 revealed no findings considered by the Panel to be treatment related. At week 13 females receiving 50 000 mg/kg diet had significantly lower creatinine, potassium and phosphorus concentrations. Urinalysis at week 6 revealed statistically increased urine volume in males receiving 15 000 mg/kg diet compared to the control; no differences between the groups were seen in the results of the urinalysis carried out at week 13.

The mean absolute pituitary weight of males receiving 15 000 mg/kg diet or 50 000 mg/kg diet and the mean absolute left adrenal weight of all male treated groups, was significantly lower than that of controls. The mean relative left kidney weight of females receiving 50 000 mg/kg diet, and mean relative right ovary weight of all female treated groups were significantly increased compared to controls. The Panel noted the absence of histopathological changes in these tissues. There was also a reduction in absolute thymus weight for males receiving the highest dose, which was also present when the weights were expressed relative to body weight but the difference was not statistically

significant. The histopathological examination of the thymus showed thymic involution/atrophy in males (1/4, 0/4, 1/4, and 2/4, at 0, 5000, 15 000 and 50 000 mg/kg diet, respectively), which is known to be a physiological change in the growing animals.

From the results of this study the concentration of 50 000 mg advantame/kg diet was considered by the authors of the study to be suitable for use in a dog study of longer duration. The Panel noted that the reduced body weight gain in the high-dose males compared to the controls could not be explained by a difference in feed intake. According to the Panel the NOAEL was 15 000 mg advantame/kg diet equal to 667 and 703 mg/kg bw/day in males and females, respectively.

In the 1-year dog study in beagle dogs (4/sex/group), advantame (99.1 to 99.2 % purity) was administered at concentrations of 0, 2000, 10 000, or 50 000 mg/kg diet (report AJO/196, 2005). Two additional dogs per sex were assigned to the control, middle and high-dose groups to be allowed to recover for 6 weeks following treatment. The dietary concentrations of advantame resulted in achieved doses of 0, 82.5, 420.9, and 2058 mg/kg bw/day in males and 0, 81.9, 406.2, and 2139 mg/kg bw/day in females. No treatment-related deaths were reported during the study but one high-dose male was sacrificed on the last day of the recovery period due to polyarteritis (i.e., the inflammation of multiple arteries also known as "beagle pain syndrome"). Pale faeces were observed in all animals in the high-dose group and in some animals from middle-dose group of both sexes. Pale faeces continued to be observed in the 50 000 mg/kg diet group through the first week of the recovery period. Loose and/or liquid faeces were noted for all animals, including controls. Body weight, body weight gain and food consumption were not statistically significantly different in the treated groups compared to the controls. Results of ophthalmoscopic or ECG examinations were not affected by treatment.

Results of the haematological examination showed statistically significantly lower mean corpuscular haemoglobin in all treated males, lower white blood cell counts in females receiving 2000 mg/kg diet, higher haemoglobin and reticulocyte count in all treated females, and higher haematocrit in females at the two highest doses. Results of the serum chemistry analyses showed significantly lower bilirubin in males at the two highest doses, higher glucose in males and cholesterol in females receiving 2000 mg/kg diet. Results of urinalysis showed higher potassium in high dose males and chloride in females at the two highest doses. None of these differences were considered by the Panel to be of toxicological significance. Relative heart weight was decreased in the high-dose male group and increased in the low-dose female group; absolute left adrenal weight was increased in the high-dose female group and absolute right ovary weight was decreased in all treated females. No treatment-related pathological changes were found. According to the Panel the NOAEL was 50 000 mg advantame/kg diet, the highest concentration tested, equal to 2058 mg/kg bw/day in males and 2139 mg/kg bw/day in females.

The results based on the 1-year dog study (report AJO/196, 2005) provided in the application dossier were published by Otabe et al. (2011c).

Considering the results of the subchronic toxicity studies overall, treatment-related decreases in body weight/body weight gain were seen at a dietary concentration of 50 000 mg advantame/kg diet in all studies apart from the 13-week study in rats and 1-year study in dogs. In the 13-week study in mice the lower body weight gain (89% of the control) was additionally recorded in the low-dose female group. The lower body weight/over all body weight gain were statistically significant in both sexes in the 4-week rat study (body weight gain 82 to 85 % of the control). In dog studies the statistical significance in body weight gain was recorded in one sex only (for females in the 4-week study (58 % of the control) and for males in the 13-week study (30 % of the control). Decrease in body weight/body weight gain after feeding laboratory animals with high doses of intense sweeteners is a known phenomenon (Chowaniec and Hicks, 1979; Grice and Goldsmith, 2000; Flamm et al., 2003; Mahew et al., 2003) associated with poor palatability and/or lower nutritional value of the diets containing high concentrations of sweeteners. However, in absence of concomitant evidence of reduced food consumption and in the presence of decreases in food consumption efficiency the decreases in body weight/body weight gain are considered likely to be adverse (Flamm et al., 2003). This is further

supported when alterations of clinical and/or post mortem pathological parameters are present at the same dose (Flamm et al., 2003).

In the subchronic rat studies several parameters of the laboratory examinations were statistically significantly altered at a dose which affected body weight/body weight gain or lower. The recorded changes although statistically significant were often slight, the dose-response was not apparent, they were not accompanied by morphological changes in any organs, and there was no consistency between the sexes in the recorded changes, between the studies in the same laboratory species or between the laboratory species. For instance, decreased haemoglobin and haematocrit, leucocyte and lymphocyte counts were recorded in the 4-week rat study at advantame dietary concentrations of 1500 mg advantame/kg diet and above and in the 13-week of 15 000 mg advantame/kg diet and above. In the dog, haematocrit and haemoglobin values were unremarkable in the 4-week study, they were decreased in the 13-week study at 15 000 mg advantame/kg diet and above, but they were increased in the 1-year study (haematocrit at 10 000 mg/kg diet and above; haemoglobin at 2000 mg/kg diet and above). In the dog, lymphocyte counts were increased in the 4-week study at 15 000 mg advantame/kg diet and above but they were not statistically significantly different from the controls in the 13-week and 1-year study. Leucocyte counts were only statistically significantly lower in the 13-week study. The Panel considered that some of the changes in laboratory parameters seen in subchronic studies, if present as isolated findings, could be considered random findings. The Panel considered however that a relationship to treatment was suggested by the fact that statistically significant changes in these parameters were seen in a relatively large number of the subchronic studies conducted, albeit not consistent from study to study. The Panel also noted that the absence of significant differences for several of the changes at the end of 4-week recovery period in the 13-week rat study could indicate a treatment-related effect. The decreases in leucocytes and lymphocytes correlated with changes in other parameters indicative of an altered function of the immune system in the 13-week study in rats (lower mitogenic lymphocytes responsiveness to Concanavalin A, lower total T-cells, lower CD4+, higher number of CD8+ cells) and with the observation of lower relative thymus weight in the 4-week rat study, and in the 4-week and 13-week (not statistically significant) dog studies in one of the sexes. In rodents, the observation of effects on lymphocytes and the reduction of the relative thymus weight in subchronic studies were considered indicative of an effect on the immune system following exposure of laboratory animals to dietary doses of advantame. The results from subchronic studies in dogs with advantame showed less clear effects on lymphocytes and thymus, although a reduction of the absolute thymus weight was noted in animals treated at the highest doses, which also affected body weight gains. The results from subchronic studies with advantame in rodents and dogs were equivocal as regards immunotoxicity and, therefore, the Panel required additional studies in young rats investigating the effects of advantame on various immunological parameters following short exposure and with groups including a post-dosing recovery phase to dose levels of 15 000 mg/kg diet and lower.

An additional study (TNO Triskelion report, 2013), investigating the potential immunotoxic effects of dietary administered advantame in young male and female Wistar rats, was submitted to EFSA. The study was conducted under GLP with albino rats. The rat was used because this species was used in previous tests with the test substance. Male and female Wistar Outbred rats (RccHanTM:WIST) were exposed for four weeks to 0, 1500, 5000 and 15 000 mg advantame/kg diet equivalent to overall mean intakes of about 140, 450 and 1380 mg/kg body weight/day, respectively. Groups of 10 rats/sex were examined directly after treatment or after a recovery period of another four weeks. Immune pathology endpoints that were studied included haematological and clinical chemistry variables, gross pathology, organ weights, histopathology of selected organs, lymphocyte subset analysis of spleen cells and proliferation and cytokine production after mitogen stimulation of spleen cells *ex vivo*. The homogeneity, content and stability of test substance in the test diets were confirmed by chemical analysis. Blood was collected from all rats at necropsy at the end of the treatment and recovery periods. General condition, growth and feed intake were not affected by the treatment. There were no treatment-related differences in total or differential white blood cell counts or in total protein concentration, albumin concentration and albumin/globulin ratio. The weights of the adrenals, spleen and thymus at the end of the treatment and the recovery period were not affected by the treatment. Macroscopic examination and microscopic examination of lymphoid organs at the end of the treatment

period did not reveal any treatment-related findings. Cell suspensions were prepared from (part of the) spleens collected at the end of the treatment period, and lymphocyte subset analysis was performed by flow-cytometry analysis. There were no treatment-related differences in lymphocyte subsets: T cells (CD3+), Helper T cells (CD3+ CD4+), Cytotoxic T cells (CD3+ CD8a+), B cells (CD45RA+), Lymphocytes (CD45+), NK cells (NKR-P1A+). The above cell suspensions were incubated with 0 and 5 μg/ml Concanavalin A (Con A) and stimulation indices were calculated. No treatment-related effects on proliferative response of splenocytes to mitogen stimulation by Con A were observed. The production of cytokines was measured in the supernatants of the Con A-stimulated splenic cell suspensions. Cytokines determined were: IFNγ (indicative for Th1 cells activity: cellular immunity), IL-4 (indicative for Th2 cells activity: antibody production and allergy), IL-10 (indicative for T regulatory cells and Th2 cells activity) and IL-2 (activates T helper lymphocytes). There were no treatment-related differences in any cytokine production after mitogen stimulation. Because no treatment-related changes were observed in the main groups, the above determinations were not conducted in the recovery groups. According to the Panel the NOAEL from this study was 1380 mg advantame/kg bw/day, the highest dose tested.

The Panel noted that the study was well designed and conducted under GLP. The statistical analysis performed was adequate. Overall, the Panel considered that this 4-week study did not reveal any immunotoxic effects in the rat.

### 3.2.3. Genotoxicity

#### 3.2.3.1. *In vitro* studies

Advantame (99.2 % purity) was tested in a bacterial reverse mutation assay with *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537, and with a tryptophan-dependent mutant of *Escherichia coli*, strain WP2*uvrA*/pKM101 (CM891) in the absence and in the presence of metabolic activation (S9 mix) (report AJO/154, 2001). Advantame was tested at concentrations of 5 to 5000 μg/plate. Results showed that advantame was not toxic at any of the concentrations tested. There was no evidence of a mutagenic potential of advantame, both in the absence and in the presence of S9 mix under the test conditions employed.

The potential of advantame (100.1 % purity) to induce forward mutations was examined in an *in vitro* mammalian cell gene mutation assay performed in subline 3.7.2c of mouse lymphoma L5178Y ( TK +/- ) cells both in the absence and in the presence of S9 mix (report AJO/159, 2002). Advantame was tested at concentrations of 39 to 5000 μg/ml. Toxicity was generally observed at concentrations of 2000 μg/ml and above. No significant increases in mutant frequencies were observed at any test point assessed. It was concluded that under the test conditions employed advantame did not demonstrate mutagenic potential.

#### 3.2.3.2. *In vivo* studies

The genotoxic potential of advantame (100.1 % purity) was examined *in vivo* based on its capacity to induce micronucleus formation in bone marrow cells of mice (report AJO/160, 2001). In this study, advantame was administered by oral gavage (20 ml/kg bw) in 1 % (w/v) aqueous methylcellulose to groups of 7 male Charles River CD-1 mice as single doses of 500, 1000, and 2000 mg/kg bw. Advantame did not produce a statistically significant increase in the incidence of micronucleated immature erythrocytes at any dose level at either the 24 or 48-hour sampling time. Compared to the vehicle controls, advantame did not result in a decrease in the proportion of immature erythrocytes at the 24-hour sampling time. There was a slight dose-related decrease in the proportion of immature erythrocytes at the 48-hour sampling time which was statistically significant at 2000 mg/kg bw, however the proportion of immature erythrocytes was within the normal laboratory control ranges. Considering the question whether advantame might have reached the bone marrow, the Panel noted that, apart from hunched postures, no other clinical signs were observed in a preliminary toxicity test and that no ADME studies in mice were available. However, based on the available results from toxicokinetic studies in rats, dogs and humans, it can be expected that advantame is also systemically

available in mice. The Panel considered that no genotoxic potential was demonstrated in the mouse micronucleus assay with advantame under the described experimental conditions.

The results based on the study reports provided in the application dossier on bacterial reverse mutation assay with *Salmonella typhimurium* and *Escherichia coli*, *in vitro* mammalian cell gene mutation assay and micronucleus formation in bone marrow cells were published by Otabe et al. (2011a).

Based on these studies there is no concern with respect to genotoxicity.

3.2.3.3. Genotoxicity of minor degradation products of advantame

β-ANS9801, β-ANS9801-acid, ANS9801-imide, and HF-1 were negative in the bacterial reverse mutation assay at concentrations of up to 5000 µg/plate in the absence and presence of metabolic activation (S9 mix) using the *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 and *Escherichia coli*, strain WP2*uvrA* tester strains (report SBL043-021, 2009; SBL043-023, 2009; SBL043-025, 2009; SBL043-027, 2009).

Negative results were obtained in the mouse lymphoma TK assay at concentrations of up to 4600, 4500, and 1000 µg/ml for β-ANS9801, β-ANS9801-acid, and HF-1, respectively, with or without metabolic activation (report SBL043-022 2009, SBL043-024, 2009; SBL043-026, 2009; SBL043-028, 2009). In the case of ANS9801-imide, positive results for induction of point mutations were observed in the mouse lymphoma TK assay when tested at concentrations of up to 500 µg/ml in the presence and absence of metabolic activation. The frequencies of both large and small colonies were increased and the global evaluation factor of 126 was exceeded at 228 µg/ml after 24-hour treatment in the absence of metabolic activation (but not after 3-hour treatment without metabolic activation) and at and above 35.1 µg/ml after 3-hour treatment in the presence of metabolic activation.

ANS9801-imide was also tested *in vivo* in the mouse micronucleus assay (report SBL043-033, 2009). At dose levels of up to 2000 mg/kg bw administered by oral gavage once daily on two consecutive days and sacrificed at approximately 24 hours after the final administration, ANS9801-imide did not increase the frequency of micronuclei in comparison to vehicle-treated control animals. The ratio of immature erythrocytes to total erythrocytes was slightly reduced at the high dose compared to control. The effect was not statistically significant; however, the ratio was less than the minimum ratio of immature erythrocytes to total erythrocytes in historical controls. This may be considered as an indication for an exposure of the bone marrow to the test substance.

The Panel noted that no genotoxicity tests were available for the metabolite HU-1. The applicant justified the absence of genotoxicity data for HU-1 based on negative genotoxicity results obtained *in vitro* with another metabolite, HF-1. According to the applicant, it is likely that the cells (*Salmonella typhimurium*, *Escherichia coli*, and mouse lymphoma cells) were also exposed in these studies to HU-1, the dealkylated metabolite of HF-1, since N-dealkylation is catalyzed by several CYP isoenzymes that would exist within the rat S9 fraction. The applicant pointed out that the N-dealkylation of HF-1 does not introduce structural alerts and that the test results on HF-1 are highly predictive of results expected with HU-1.

Based on these data and considering that ANS9801-imide did not show genotoxicity in vivo and HU-1 is only a minor metabolite, the Panel concluded that there is no concern with respect to genotoxicity of advantame metabolites.

### 3.2.4. Chronic toxicity and carcinogenicity

The chronic toxicity and carcinogenicity of advantame was studied in mice and rats.

In a carcinogenicity study Crl:CD-1 (ICR) BR mice (64/sex/group) received 0, 2000, 10 000, or 50 000 mg/kg diet of advantame (99.3 % purity) for 104 weeks (report AJO/198, 2006). The dietary concentrations of advantame resulted in achieved doses of 0, 213, 1057, and 5693 mg/kg bw/day in

male animals and 0, 272, 1343, and 7351 mg/kg bw/day in female animals. There was no effect of treatment on mortality. The survival at week 104 was 31 % (20/64), 27 % (17/64), 34 % (22/64), 38 % (24/64) in males, and 41 % (26/64), 23 % (15/64), 33 % (21/64), 27 % (17/64) in females, at the 0, 2000, 10 000, or 50 000 mg/kg diet of advantame, respectively. There was no effect of treatment on mortality. Although survival was somewhat low at terminal sacrifice, the Panel found it acceptable considering the duration of the study, and adequate for assessment of carcinogenic potential. Body weight gain in the high-dose group females, but not in high-dose males, was statistically significantly lower than controls over the 104-week treatment period (76 % of control). A trend towards lower body weight was also observed for male mice, however these differences failed to achieve statistical significance. Food consumption was not affected by treatment. A slight, statistically non-significant decrease in food conversion efficiency was reported in both sexes receiving 50 000 mg/kg diet (12.5 % and 13.4 % of decrease, in male and female, respectively) compared to the controls. The only haematological parameter statistically significantly different from the control was a lower erythrocyte count at the high dose in both sexes (males 7.27 vs 8.48, $p < 0.01$; females 6.85 vs 7.68, $p < 0.05$). All other haematological parameters and absolute and relative organ weights were not statistically significantly different from the controls. The Panel noted that the haematological examination did not include all parameters usually examined and was performed only at the termination and that clinical chemistry and urinalysis was not included in the study. There were no treatment-related non-neoplastic findings. The malignant tumours for which statistically significantly increased incidences were recorded in some of the treated groups included bronchio-alveolar adenocarcinoma (males: 11/64, 14/64, 6/64, 6/64; females: 1/64, 7/64 ($p < 0.05$), 5/64, 5/64, at 0, 2000, 10 000, and 50 000 mg/kg diet, respectively) and histiocytic sarcoma of the haematopoietic system (males: 0/64, 5/64 ($p < 0.05$), 0/64, 1/64; females: 3/64, 1/64, 4/64, 1/64 at 0, 2000, 10 000, and 50 000 mg/kg diet, respectively). For both tumours the Panel noted the absence of a dose-response relationship in both sexes and that the incidences were within historical control ranges for CD-1 mice between 78-104-week old. Thus the Panel considered these tumours not to be treatment-related. The Panel further noted that the pulmonary tumours are known to have a high background incidence in mice. The incidence of harderian gland adenoma (males: 1/1 examined, 2/5, 3/5, 6/6; females: 2/5; 1/3, 1/1, 1/3 at 0, 2000, 10 000, and 50 000 mg/kg diet, respectively) was higher in treated males compared to the control group. As the incidence was within the range for historical control the Panel considered that this was not a treatment related but a random finding. Overall, the Panel considered that none of the reported neoplastic findings were related to treatment with advantame. Accordingly the Panel considered that the results indicated no carcinogenic potential of advantame at doses up to 50 000 mg/kg diet, the highest concentration tested, and equal to 5693 and 7351 mg/kg bw/day in males and females, respectively. With regard to the lower body weight gain in the high-dose group the NOAEL was 10 000 mg advantame/kg diet, equal to 1057 and 1343 mg advantame/kg bw/day in males and females, respectively.

In a combined chronic toxicity and carcinogenicity study Han Wistar rats (55/sex/group) were administered advantame (98.9 to 99.8 % purity) in the diet at concentrations of 0, 2000, 10 000, or 50 000 mg/kg diet for 104 weeks in order to assess the toxic and carcinogenic potential of advantame (report AJO/195, 2006). Additional groups (20/sex/group) were dosed for 52 weeks to assess chronic toxicity. Additional groups (10/sex/group) were assigned to the control 10 000 and 50 000 mg/kg diet group and were treated for 52 weeks followed by a 6-week recovery period. Further groups (12/sex/group) were assigned to each of the treatment groups (except controls) to form the satellite study designed to investigate plasma concentrations of advantame and to provide blood for haematological and blood chemistry examinations. All of the animals were the offspring of parental animals, which had been provided the respective dose levels for 4 weeks prior to pairing, through gestation, and on to the 21$^{st}$ day post-birth (weaning). Over the 2 years of study, the dietary concentrations of advantame resulted in achieved doses of 0, 97, 488, and 2621 mg/kg bw/day in males and 0, 125, 630, and 3454 mg/kg bw/day in females. There was no effect of treatment during the *in utero* phase on mortality, maternal body weight gain and food consumption, fertility, or on the growth and survival of offspring. During the treatment period for the carcinogenicity phase, the percentages of survival were 69 % (38/55), 69 % (38/55), 71 % (39/55), 71 % (39/55) for males, and 65 % (36/55), 60 % (33/55), 55 % (30/55), 53 % (29/55) for females, at the 0, 2000, 10 000, and

50 000 mg/kg diet, respectively. A few clinical signs (i.e. pale anus and pale or discoloured faeces) were found at the middle and the high dose but resolved by the end of the recovery period.

Body weight gain in the high-dose males was slightly reduced (95 % of control) compared to controls after 52 weeks of treatment in the toxicity phase, but it did not reach statistical significance. During the 6-week recovery phase, the body weight gain of males (162 % of control) and females (150 % of control) was increased compared to that of controls, attaining statistical significance in males. As with the 52-week toxicity phase, the body weight gains in the males (93 % of control) and females (94 % of control) treated at 50 000 mg/kg diet for 104 weeks were slightly reduced, attaining statistical significance in males only. While treatment-related, the applicant considered this effect on body weight not to be of toxicological significance, but due to the non-nutritive nature of the high dietary concentration of advantame. During the toxicity and carcinogenicity phase, food consumption was slightly increased in males (104 % of control) and females (108-109 % of control), while food conversion efficiency was slightly decreased in both sexes (9 % of control), at the 50 000 mg/kg diet dose level. Water consumption after the toxicity phase was slightly increased in treated males and females compared to the control, reaching statistical significance only in females at 2000 mg/kg diet (40 vs 34 ml/rat/day; $p < 0.05$). Results of haematological investigations showed statistically significantly decreased prothrombin time in high-dose males after 52 weeks, and a decrease in large unstained cells for high dose males after both 52 and 104 weeks of treatment. In females there was a lower monocyte count at the high dose after 52 weeks of treatment. The blood chemistry analyses in males revealed lower alkaline phosphatase, urea, and sodium concentrations at the high dose, and a lower α 1-globulin at 10 000 mg/kg diet after 52 weeks; in addition, a decrease in urea and an increase in sodium were observed in the high-dose group after 104 weeks. In females, there were a lower α 1-globulin, and increased potassium and chloride at the high dose after 52 weeks of treatment. Urinalysis data revealed, after 52 weeks, a statistically significant increase in specific gravity and total sodium in high-dose treated males, and for females an increase in protein at 50 000 mg/kg diet and in total sodium at 10 000 mg/kg diet and above. The Panel noted that the changes in laboratory investigation parameters were slight, with no apparent dose-response, and generally present only in one sex.

Organ weights showed several statistical significant differences from the controls. After 52 weeks high-dose males had higher absolute and relative pituitary weights and middle dose group males had higher absolute lung and bronchi weight. For middle-dose females at 52 weeks there was a higher absolute kidney weight and at the highest dose absolute uterus and cervix weights were lower than those of controls. After 104 weeks, significant changes in organ weights compared to the controls were found mainly in males. These were higher absolute kidney weight at the low dose, higher absolute and relative liver weights at the middle dose, higher absolute weights of the heart and of seminal vesicles, and higher relative weights of the brain, epididymis, liver, lungs and bronchi, and salivary gland at the high dose. In females a higher absolute brain weight and higher absolute and relative weights of salivary glands were recorded.

No treatment-related macroscopic or non-neoplastic histopathological findings were found in the study. A higher incidence of pancreatic islet-cell carcinomas in high-dose males (incidence rates of 0/55, 1/55, 2/55 and 3/55 in the control through high-dose male groups, respectively) or the incidence of pancreatic islet-cell carcinoma and adenoma combined (incidence rates of 2/55, 5/55, 4/55 and 5/55 in the control through high-dose males) failed to achieve statistical significance (carcinoma; pairwise comparison $p = 0.134$, trend test $p = 0.06$; adenoma and adenocarcinoma combined pairwise comparison $p = 0.221$, trend test $p = 0.263$). The Panel noted that the pancreatic tumour incidences generally remained within background historical control values, provided by the authors of the study. In the high-dose females the incidence of mammary gland adenomas was statistically significantly higher than that of the concurrent controls but it was within the incidence rates of the historical control (incidence rates of 0 in the control through 10 000 mg/kg diet groups and 4/41 in the 50 000 mg/kg diet group). Mammary adenocarcinomas were present in 3/43, 1/44, 3/42, and 5/41 of the control through high-dose females, respectively (statistically not significant). Combining the incidence of mammary gland adenomas and adenocarcinomas (3/43, 1/44, 3/42 and 9/41 in the control through the high-dose female) no pairwise comparisons achieved statistical significance. Combining the incidence

of mammary gland adenoma, fibroadenoma and adenocarcinoma revealed a positive trend test ($p = 0.015$); however no pairwise comparisons achieved statistical significance. Combining the benign mammary gland adenomas and the fibroadenomas (incidence rates of 6/43, 8/44, 9/42 and 12/41) resulted in a positive trend test ($p = 0.026$) and a significant pairwise comparison ($p = 0.042$) at the 50 000 mg/kg diet dose level. The Panel noted that while combining the incidence of adenoma and adenocarcinoma is a common practice, helpful in considerations of a carcinogenic effect of a test compound, as both tumours originate from the same cellular structures, combining incidence of fibroadenoma with those of adenoma and adenocarcinoma is not a common practice and provides little information Since the incidence rates for all the mammary gland tumour types and combinations were within the historical control range (as informed by the authors of the study), with the control values near the bottom of the range and the values in the high-dose group being near the top of the range, and as mammary tumours belong to the background pathology of aging female rats the increased incidences in this study were not considered by the Panel to be treatment-related. According to the Panel the results indicated no carcinogenic potential of advantame in doses up to 50 000 mg/kg diet, the highest concentration tested, and equal to 2621 and 3454 mg/kg bw/day in males and females, respectively.

The results based on combined chronic toxicity and carcinogenicity study in rats (report AJO/195, 2006) provided in the application dossier were published by Otabe et al. (2011b).

Based on the carcinogenicity studies in mice and rat, the Panel concluded that there is no concern with regard to carcinogenicity of advantame. At the high doses of advantame in the carcinogenicity studies a lower body weight gain was recorded in mice achieving statistical significance for females compared to the controls. In rats body weight gain in the high-dose males was slightly lower after 52 weeks, but the difference from the controls was not statistically significant. After 104 weeks the body weight gains of males and females were slightly reduced, attaining statistical significance in males only.

### 3.2.5. Reproductive and developmental toxicity

Reproductive and developmental toxicity studies were conducted in rat and rabbit.

In a two-generation reproduction toxicity study advantame (98.6 % purity) was administered at concentrations of 0, 2000, 10 000, or 50 000 mg/kg diet to Charles River CD Sprague-Dawley IGS BR rats (30/sex/group) for 10 weeks prior to pairing, and then through pairing, gestation, lactation, until termination (report AJO/203, 2004). The $F_1$ generation (25/sex/group) continued to receive the same dietary concentrations as the parental animals from the $F_0$ generation. The mean achieved doses for $F_0$ generation were reported: 164, 833, and 4410 mg/kg bw/day in males prior to pairing; 184, 907, and 4776 mg/kg bw/day, 163, 795, and 4136 mg/kg bw/day, and 320, 1575, and 8192 mg/kg bw/day in females prior to pairing, during gestation, and during lactation, respectively. For $F_1$ generation the advantame doses were reported: 204, 1036, and 5431 mg/kg bw/day in males prior to pairing; 229, 1139, and 5920 mg/kg bw/day, 167, 865, and 4457 mg/kg bw/day, and 316, 1592, and 8447 mg/kg bw/day in females prior to pairing, during gestation, and during lactation, respectively. There was no treatment-related mortality in the $F_0$ animals or in the $F_1$ generation. There were no clinical signs of overt toxicity or treatment-related effects on any of the parameters measured. The only remarkable finding was of changes in the colour of the faeces during treatment. Body weights, body weight gains, and food consumption were unaffected by the treatment. Food consumption and food conversion efficiency, at the high dose, were, in some cases, slightly higher and lower respectively. There were no effects of treatment on any of the parameters measured in the $F_0$, $F_1$, or $F_2$ generations i.e. clinical signs of toxicity in $F_1$, sensory examination in $F_1$ such as motor and neuromuscular function, sexual maturation in $F_1$, necropsy observations in $F_0$ and $F_1$ parents such as sperm parameters and organ weights in $F_0$, and $F_1$ such as adrenals, brain, epididymis, kidneys, liver, ovaries, pituitary, prostate, seminal vesicles, spleen, testes, and uterus and histopathological examination in $F_0$ and $F_1$ parents of adrenals, epididymis, mammary area (females with total litter loss only), ovaries, pituitary, prostate, seminal vesicles, testes, uterus, vagina, and $F_1$ and $F_2$ pups at necropsy brain, spleen and thymus weights. The NOAEL for reproductive toxicity was 50 000 mg advantame/kg diet ($F_0$ before mating:

4410 mg/kg bw/day in males, 4776 mg/kg bw/day in females; $F_0$ during gestation: 4136 mg/kg bw/day; $F_1$ before mating: 5431 mg/kg bw/day in males, 5920 mg/kg bw/day in females; $F_1$ during gestation: 4457 mg/kg bw/day), the highest concentration tested.

The results based on the two-generation reproduction toxicity study (report AJO/203, 2004) provided in the application dossier were published by Otabe et al. (2011e).

In a dietary prenatal developmental toxicity study, advantame (100.1 % measured purity) was administered at concentrations of 0, 5000, 15 000, or 50 000 mg/kg diet to groups of 22 female Charles River CD Sprague-Dawley IGS BR rats from day 0 to day 20 after mating (inclusive) (report AJO/182, 2002). Maternal performance, *in utero* survival, growth, and morphological development of the offspring throughout gestation were evaluated. The females were killed on gestation day (GD) 20 and their uterine contents examined. The dietary concentrations of advantame resulted in achieved doses of 0, 465, 1418, and 4828 mg/kg bw/day. There were no mortalities and no clinical signs of overt toxicity. Treated animals showed changes in the colour and form of the faeces during treatment. There was no treatment-related effect on mean absolute body weight. Mean body weight gain at 50 000 mg/kg diet was statistically significantly lower than the control (30, 23, 16, 13, 11, and 9 % lower at GD 0-3, 0-6, 0-10, 0-14, 0-18 and 0-20, respectively). When adjusted for gravid uterine weight, mean body weight gain from GD 0-20 in the high-dose group was slightly but statistically significantly lower (~16 %) compared to the controls. In the high-dose group mean food consumption was statistically significantly lower than the control during the first two GDs but thereafter it was statistically significantly higher (~7 % during GD 10-13 and 14-17). No signs of toxicity occurred in dams treated at 15 000 mg advantame/kg diet. No treatment-related effects were found with respect to the number of implantations, resorptions, live fetuses, pre and post-implantation losses, sex ratio, and placental, fetal, and total litter weights. The Panel considered the effect on maternal body weight gain treatment-related and indicative of toxicity of the high dose of advantame. Hence the 15 000 mg advantame/kg diet equal to 1418 mg/kg bw/day was considered to be the NOAEL for maternal toxicity and 50 000 mg advantame/kg diet equal to 4828 mg/kg bw/day was considered a NOAEL for developmental toxicity.

Advantame (mean concentration from 99.5 to 102 % in the formulation tested) was administered daily by oral gavage at doses of 500, 1000, or 2000 mg/kg bw/day from day 6 to 28 of gestation to groups of 24 mated female New Zealand White rabbits (report AJO/190, 2003). Control animals received the vehicle, 1 % methylcellulose. The doses for this study were chosen based on the results of a preliminary developmental toxicity study in the rabbit by oral gavage administration (report AJO/183, 2005).

All animals that died or aborted and those animals that survived to GD 29 after mating were subjected to a detailed necropsy. Examination of uterine contents was performed for litters surviving to GD 29 and the fetuses were examined for external and visceral changes followed by skeletal examination after staining of the skeleton. Developmental toxicity was assessed by evaluation of *in utero* survival, growth and morphological development of the fetuses on GD 29.

Almost all animals receiving advantame showed coloured staining of the under-cage tray paper and sporadic observations of green/pink colouration of the urine were recorded. Treatment at 2000 mg/kg bw/day was associated with a lower body weight gain to GD 10, however body weight gain by GD 29 was essentially similar to that of controls. At lower dosages there was no clear effect of treatment on body weight gain. A total of 1, 0, 1 and 5 animals from the groups 0 (Control), 500, 1000 and 2000 mg/kg bw/day respectively were killed before scheduled necropsy for reasons of animal welfare. Five of the 24 (21 %) mated females rabbits from the 2000 mg/kg bw/day group, sacrificed prematurely (between days 17 -27) for animal welfare reasons, manifested high bodyweight loss (from 200 to 570 g) and a range of clinical signs and necropsy findings that suggested compound related toxicity. In addition to general staining of different body fluids and tissues, the animals showed reduced body temperature, decreased water intake and vocalization and overactive behaviour suggesting acute toxicity. Upon necropsy consistent findings such as large amounts of air and stained

fluids and material in the caecum, in the GI tract and in the stomach as well as few soft faecal pellet formation in the rectum were reported. In some animals, either pale liver or liver with a pronounced lobular pattern, an enlarged gall bladder, multiple cysts in both kidneys and covering of the entire wall of the caecum with haemorrhagic gelatinous material were diagnosed. In the 1000 mg/kg bw/day group one animal out of twenty-four (5 %) had to be prematurely sacrificed and presented similar findings as those previously reported at the highest dose: high weight loss (710 g), decreased water intake, staining of fluids and tissues, loose faeces and stained faeces, difficulty to breath, vocalization, loss of locomotion and coordination. Necropsy examination showed additionally to the staining of some organs, kidneys with multiple punctuate foci on the surfaces, caecum and stomach walls covered with gelatinous material, caecum wall haemorrhagic and part of the stomach walls adjacent to the pyloric sphincter thickened. In the control group two animals out of twenty-four (10 %) were prematurely sacrificed during the study, however although one of those animals showed also decrease water intake, convulsion, difficulty to breath and vocalization, upon necropsy examination none showed the same effects in the gastrointestinal tract as those reported in the treated groups.

There were 3, 6, 2 and 6 non-pregnant animals at 0 (Control), 500, 1000 and 2000 mg/kg bw/day, respectively and this resulted in totals of 19, 18, 21 and 12 animals respectively with live fetuses at termination on GD 29 of gestation at these dosages. Litter data as assessed by the number of early, late and total resorptions, implantations and live fetuses and sex ratio (percentage male), were not clearly affected by treatment with advantame at any of the doses investigated. There was no clear adverse effect of treatment upon group mean values of placental, litter and fetal weights in any of the treatment groups. The intergroup variations observed in litter weights were considered to reflect the variation in group mean litter size between treated groups.

At 2000 mg/kg bw/day group, there was a suggestion of an increase in the incidence of fetal deaths (late resorptions). Although this was not conclusively attributable to treatment, it was noted that the animal which aborted one fetus very late in pregnancy (GD 29) had a high proportion of dead fetuses *in utero* (i.e. three of the remaining four fetuses were dead). In addition, it should be noted that the number of live fetuses per litter was comparable to the control group and within the historical control range for this strain of rabbits. According to the authors neither the type, incidence or distribution of major or minor visceral or skeletal abnormalities or the incidence of skeletal variants indicated an adverse effect of treatment with advantame. At 1000 mg/kg bw/day group, four fetuses from the same litter and one from another litter were observed to have major head abnormalities. The malformations seen in four fetuses from one litter were: microcephaly, agnathia, lack of face features, hydrocephaly, absent olfactory lobes and fused frontal lobes, multiple heart defects, one side eye defect (folded retina), multiple heart defects; unilateral microphthalmia, multiple heart defects. The malformations of one fetus from another litter from the same dose group were: domed cranium, exophthalmia, syndactyly hindlimb. Acephaly was seen in one fetus from the control group. The Panel noted that although no data for historical control incidence of abnormalities have been submitted, the head abnormalities recorded at 1000 mg/kg bw/day could be coincidental and not a treatment-related finding, as most of these abnormalities were related to one litter and there were no similar head abnormalities in the twelve litters from the 2000 mg/kg bw/day group. The Panel considered 500 mg advantame/kg bw/day to be the NOAEL for maternal toxicity based on disturbances of gastrointestinal tract causing morbidity that required killing the animals for welfare reasons and 1000 mg advantame/kg bw/day to be the NOAEL for developmental toxicity.

The results based on the prenatal developmental toxicity studies in rats and rabbits (AJO/182, 2002; AJO/190, 2003) provided in the application dossier were published by Otabe et al. (2011d).

### 3.2.6. Other studies

#### 3.2.6.1. Human studies

The human tolerability of advantame has been tested in three clinical studies.

In a single-dose oral tolerance study, 24 healthy male volunteers aged between 18 and 40 years old consumed oral doses of 0.1, 0.25, or 0.5 mg advantame/kg bw as an aqueous solution (150 ml) (report ANSE-101 (AJO/209), 2003; Warrington et al. 2011). The ingestion of advantame was well tolerated with no reports of severe adverse effects or withdrawals during the study. The reported adverse events (i.e. infections and infestation, nervous system, respiratory, thoracic and mediastinal disorders) were mild in intensity and were considered to be unrelated to the study treatment. Furthermore, there were no significant changes in vital signs (i.e. systolic and diastolic blood pressure or pulse rate) reported in the study subjects.

A safety and tolerability assessment of multiple daily doses of advantame was performed in 24 normal healthy males and females subjects (aged between 16 and 55 years old) who consumed repeated doses of 10 mg advantame as single capsules (3 times daily) for a period of 4 weeks (report ANSE-103a, 2003 ; Warrington et al. 2011). The daily dose corresponded to 0.375 to 0.5 mg advantame/kg bw/day for subjects with body weight between 60 and 80 kg. The administration of advantame was reported to be generally well tolerated throughout the experimental period. Three mild adverse events were reported: two subjects in the advantame group reported the development of pruritis, one of which was considered possibly related to treatment; one case of viral respiratory tract infection was reported in a placebo subject. No severe adverse events were noted during the study. Advantame did not affect plasma levels of glucose or insulin in these normal subjects, and no change in the glucose/insulin ratio was observed between treatment groups. The statistical analysis of the effects of treatment, gender, time, and interactions between these variables revealed a limited number of significant effects of treatment (mainly related to systolic blood pressure, blood urea nitrogen, calcium, phosphate, cholesterol and basophils); however, none were considered of biological significance and were considered likely to be random findings. The Panel concurred with this opinion.

Another safety and tolerability assessment of multiple daily doses of advantame was performed in 72 subjects (36 males and 36 females; aged 60.1 ± 7.3 years) with type II diabetes who consumed repeated doses of 10 mg advantame as single capsules 3 times daily for a period of 12 weeks, which corresponded to 0.375-0.5 mg advantame/kg bw/day for subjects with body weight between 60 and 90 kg, (report ANSE-103b, 2006). The administration of advantame was reported to be well tolerated. Fourteen subjects (5 in the advantame group and 9 in the placebo group) reported 19 adverse events, most of which were related to various infections (bronchitis, cystitis, naso-pharyngitis, and Urinary Tract Infection (UTI)), and were reported at approximately equal frequency in both groups. Three adverse events (flatulence, dyspepsia, and nausea) of moderate severity in one subject were considered by the authors of the study to be possibly due to advantame treatment. With the exception of the UTI all adverse events resolved by the end of the study. With the exception of increased urinary mean occult blood levels (at each study visit for subjects in the treatment group), biochemical, haematological, and urine analyses were unremarkable as no significant treatment-related changes were reported. According to the authors of the study the increase in urinary occult blood levels in the advantame group was determined not to be due to advantame treatment, but due to incorrect urine collection procedure in a small number of female subjects. No significant effect of treatment was observed on blood pressure and heart rate. Advantame did not aggravate glucose intolerance or insulin resistance in the 24 sub-group test subjects (12 males and 12 females, aged 60.1 ± 7.3 years). Advantame was well tolerated and had no impact on glucose homeostasis following the consumption of high doses over a 12-week period in diabetic subjects.

In the single-dose clinical trial advantame was well tolerated in single doses of up to 0.5 mg/kg bw/day. Any adverse events reported by the participants were mild and unrelated to the consumption of advantame. Similarly, in the two multiple-dose clinical studies the consumption of repeated doses of advantame of up to 0.5 mg/kg bw/day for 12 weeks was well tolerated by both normo-glycemic individuals and diabetics.

3.2.6.2. Special studies

*Irwin dose-range study on behavioural aspects in rats*

Male Han Wistar rats (6/group) were administered advantame at doses of 10, 100, or 1000 mg/kg bw (99.9 % purity), or an equivalent volume of the vehicle (1 % w/v methylcellulose), by oral gavage and monitored for 7 days post-dosing for behavioural and physiological changes (report AJO/161, 2001). No behavioural or physiological alterations were observed and no mortality resulted from the administration of advantame.

*Assessment of locomotor activity in rats*

Male Han Wistar rats (10/group) were administered advantame at doses of 10, 100, or 1000 mg/kg bw (99.9 % purity), or an equivalent volume of the vehicle (1 % w/v methylcellulose), or 3 mg amphetamine sulphate/kg bw (positive control) (report AJO/162, 2001). The locomotor activity was measured for 10 minutes at each interval, beginning with the pre-dosing period, and followed by measurements up to 300 minutes post-dosing. The locomotor activity of rats was unaffected by the administration of advantame compared with vehicle-treated animals. As positive controls, rats were administered amphetamines and were reported to have significantly higher locomotor activity compared with the vehicle-treated animals.

*Cardiovascular and respiratory evaluation in the dog*

Male beagle dogs (3/group) were administered advantame (99.9 % purity) at doses of 10, 100, or 1000 mg/kg bw or the dosing vehicle (1 % w/v methylcellulose) (report AJO/163, 2001). The authors of the study reported that the duodenal administration of advantame resulted in no significant effects on general respiratory status, resistance of the peripheral vasculature, and or on electrical status of the myocardium compared with the vehicle control group. Intra-duodenal administration of advantame produced no biologically meaningful effects on cardio-respiratory parameters. However, the 100 mg advantame/kg bw dose was reported to result in a statistically significant reduction in mean arterial blood pressure at the 120, 150, and 165 minute sampling times post-dose. Although statistically significant, this finding was not considered to be of biological significance by the authors of the study.

*Gastrointestinal motility study in rats*

Male Han Wistar rats (10/group) were administered advantame (99.9 % purity) at doses of 10, 100, or 1000 mg/kg bw by oral gavage, or an equivalent volume (4 ml/kg) of the vehicle (1 % w/v methylcellulose) or 10 mg morphine sulphate/kg bw (positive control) to assess effects of advantame on gastrointestinal motility in the charcoal propulsion test (report AJO/164, 2001). No significant effects were reported on the gastrointestinal motility of rats treated with 10 or 100 mg advantame/kg bw compared with vehicle-treated controls. The ingestion of 1000 mg advantame/kg bw was reported to result in a moderate statistically significant decrease in gastrointestinal motility compared to rats administered the vehicle. The authors of the study attributed this effect to the physical properties of the dosing formulation (i.e. the bulking effect) rather than to the pharmacological effect of the test material.

3.2.6.3. Potential toxicity of the major and minor degradation products of advantame

A number of major and minor degradation products of advantame have been identified.

*Major degradation product of advantame: ANS9801-acid*

ANS9801-acid was identified as the principal degradation product of advantame and a known impurity, with its presence limited to less than 1 % in the final product. In addition, ANS9801-acid also is the major metabolite of advantame and has been identified in the plasma, urine, and faeces of all tested species (humans, dogs, and rats). Following consumption, advantame is rapidly converted to

ANS9801-acid in the gut prior to absorption. Advantame that is absorbed prior to metabolism to the acid is converted to ANS9801-acid in plasma. The applicant did not submit any toxicological test in support of the safety of ANS9801-acid. The applicant explained this choice indicating that, since ANS9801-acid is available systemically following ingestion of advantame as a result of its presence as an impurity of the final product and from the metabolism of advantame to the acid, the series of toxicological studies conducted mainly to assess the safety of advantame also can be relied upon to support the safety of ANS9801-acid.

*Minor degradation product of advantame:*

β-ANS9801, β-ANS9801-acid, ANS9801-imide, HF-1, and L-phenylalanine methyl ester were identified as the minor degradation products of advantame in a study in mock beverages with advantame at concentration of 50 mg/l. L-Phenylalanine methyl ester is expected to hydrolyse to L-phenylalanine and methanol; therefore further studies were not conducted to assess its safety. The safety of the remaining degradation products (β-ANS9801, β-ANS9801-acid, ANS9801-imide, and HF-1) was assessed individually in a series of *in vitro* genotoxicity assays (see section 3.2.3.3).

While β-ANS9801, β-ANS9801-acid, ANS9801-imide, and HF-1 were identified in the mock beverage study as minor degradation products of advantame, the test beverages were prepared with advantame added at a level of 50 mg/ml, which exceeds the actual expected use level of advantame in carbonated beverages by approximately 4-fold. Considering the results of the *in vitro* and *in vivo* genotoxicity studies (section 3.2.3.3), as well as the low levels likely to be present in the diet, the minor degradation products of advantame, β-ANS9801, β-ANS9801-acid, ANS9801-imide, and HF-1, are not expected by the Panel to be associated with any adverse effects following oral consumption of advantame by humans under the proposed conditions of use.

Another degradation product to which consumers would be exposed via consumption of advantame-containing products is methanol. Methanol released by advantame hydrolysis in the gastrointestinal tract represents 7 % by weight of advantame. Based on the exposure estimates for advantame reported in Table 4 in the five European population groups, the estimated exposure to methanol from all food and beverage application of the sweetener would be approximately (minimum mean up to maximum 95$^{th}$ percentile) from 0.001 up to 0.020 mg/kg bw /day corresponding to 0.07-1.4 mg/person/day for an adult[9] and from 0.005 up to 0.052 mg/kg bw/day corresponding to 0.06-0.6 mg/person/day for a toddler. The methanol content in the advantame sweetened water-based beverages at the maximum proposed level of use (12 mg advantame/l) is estimated to be 0.84 mg/l under the assumption that hydrolysible methanol is 7% by weight of advantame. In comparison methanol concentration of fruit juices ranges from 1-640 mg/l with an average of 140 mg/l. Methanol is produced endogenously at the rate of approximately 300-600 mg/day (COT, 2011). In addition to endogenously produced methanol and methanol present in the diet, the methanol from ingestion of advantame sweetened products represents a small addition to the overall methanol exposure and is considered by the Panel to be of no safety concern.

The Panel estimated the amount of phenylalanine to which consumers might be exposed via ingestion of advantame as a general purpose sweetener based on the assumption that all ingested advantame can be metabolised to ANS9801-acid, which thereafter can be metabolised to phenylalanine. Under these circumstances phenylalanine represents 35.8 % by weight of advantame. Based on the exposure estimates for advantame reported in Table 4 in the five European population groups, the estimated exposure to phenylalanine from all food and beverage application of the sweetener would be approximately (minimum mean up to maximum 95$^{th}$ percentile) from 0.004 up to 0.1 mg/kg bw/day corresponding to 0.3-7 mg/person/day for an adult and from 0.02 up to 0.26 mg/kg bw/day corresponding to 0.2-3.1 mg/person/day for a child.

[9] A body weight of 70 kg for adult and 12 kg for toddler were used according to EFSA Scientific Committee (2012).

Phenylalanine is an essential amino acid and therefore must be supplied by the diet. The recommended aromatic amino acid requirement (phenylalanine or tyrosine) is set at 25 mg/kg bw/day (WHO, 2007). In the US, the Institute of Medicine sets estimated average requirement (EAR) for amino acids and the phenylalanine plus tyrosine EAR for adults is estimated at 27 mg/kg bw/day (IOM, 2005). The mean daily consumption of phenylalanine in the US is estimated to be 3.4 g/day (IOM, 2005). A French study (Rousseau et al., 2006) reported in an AFSSA report on protein intake (AFSSA, 2007) estimated the daily intakes of different populations, from sedentary people to sportspersons (several levels of expenditure of physical energy). The mean phenylalanine intake for sedentary people was estimated to be 3.8 g/day.

Additional information on phenylalanine intake from natural food occurrence through the diet and phenylalanine content per food category of the EFSA Comprehensive Database was provided to EFSA (Egermann et al., unpublished data). Concentration data of phenylalanine in foods came from the German food composition database (Bundeslebensmittelschlüssel). The levels of phenylalanine content per food category (according to level 2 of the food additives nomenclature from Regulation No 1129/2011) range from 0.01 mg/g in wine to 31.05 mg/g in meat substitutes; some food groups do not contain any phenylalanine. These levels linked to the EFSA Comprehensive Database allow calculation of phenylalanine intake from the diet. These intakes range from 58 mg/kg bw/day for adults to 137 mg/kg bw/day for children at the mean within the EU population.

The daily dietary exposure to phenylalanine of a phenylketonuric homozygous child (20 kg) on a phenylalanine restricted diet has been reported to range from 400 to 600 mg/person/day (corresponding to from 20 to 30 mg/kg bw/day) (FDA, 2002). The Panel noted that the exposure to phenylalanine from advantame represents a small increment (< 1 %) in the exposure of the phenylketonuric homozygous child to this amino acid.

#### 3.2.6.4. Evaluation of the potential nitrosation of advantame

Since advantame is a secondary amine, the applicant evaluated its potential to participate in the formation of *N*-nitroso compounds in foods and beverages (e.g., soft drinks and fruit-flavoured juices), where advantame may interact with the nitrite ion in the beverages to form nitrosamines, and in the stomach, where the acidic environment and the presence of nitrite provide a suitable site for nitrosation of advantame. The hypothetical amount of nitrosamines formed from the consumption of advantame-sweetened foods was reported to be in the range of picogram/kg, compared with other *N*-nitroso compounds detected in foods which were in the range of μg/kg (Tricker and Preussman, 1988; Tricker and Kubacki, 1992). However, the exact amount of *N*-nitroso advantame formed in beverages and in the human stomach is unknown. Nevertheless, the applicant stated that it is likely to be less than the hypothetical worst-case calculation provided, based on a worst-case assumption that all ingested advantame undergoes *N*-nitrosation; no inhibitors of *N*-nitrosation are present in the diet; and all *N*-nitroso advantame is absorbed. The applicant reported that the risk of cancer from the *N*-nitrosation of advantame in the human stomach under normal physiological and dietary conditions will be likely to be further reduced by the presence of certain inhibitors of *N*-nitrosation in carbonated soft drinks and other foods (i.e. the preservative sodium benzoate, or ascorbic acid). The toxicological results of the mouse and rat carcinogenicity studies provide further assurances regarding the safety of advantame and the very low risk presented by the *N*-nitrosation of advantame. Overall, the Panel considered that the potential formation of *N*-nitroso advantame does not raise a safety concern.

## 4. Discussion

The present opinion deals with the safety of advantame for use as a high-intensity sweetener in various food and tabletop products.

Advantame is described by the applicant as an *N*-substituted (aspartic acid portion) derivative of aspartame. The final advantame product is described as having a minimum of 97 % purity (on an anhydrous basis). The Panel noted that in several non-consecutive batches of the final product, the levels of platinum and palladium, residues from the catalysts used in the catalytic hydrogenation

process, could amount up to 1.7 and 5.3 mg/kg, respectively, and therefore, the Panel considered that a maximum limit for palladium and for platinum should be included in the specifications.

The advantame bulk material has been demonstrated to be stable following storage for up to five years under normal storage conditions and up to six months under accelerated conditions. The stability of advantame has been also investigated under intended conditions of use in foods and non-alcoholic beverages. The Panel noted that in heat-treated beverages it is reported that about 50 % of advantame is degraded. A similar result has been reported in yellow cake, where approximately 39 % of advantame is degraded during batter preparation and baking, and production of ANS9801-acid occurs. The Panel noted that there was an indication of advantame instability in acidic beverages and thermally treated foods.

Since advantame is reported to be approximately 37 000 times sweeter than sucrose, the applicant indicated that the intended use of advantame within the EU market is to replace caloric sugars (sucrose, glucose, fructose, etc.) in the categories of foods in which high-intensity sweeteners are authorised according to Annex II to Regulation (EC) No 1333/2008 on food additives.

Metabolism and pharmacokinetics of advantame and its main metabolite, ANS9801-acid, have been studied in rats, dogs, and in humans.

Overall the metabolism and toxicokinetics studies show that orally administered advantame is rapidly converted in the gastrointestinal tract to ANS9801-acid which can be rapidly absorbed. However, the bioavailability of the ANS9801-acid is limited. Following i.v. administration, advantame is rapidly converted to the ANS9801-acid. Following oral dosing of radiolabelled advantame, radioactivity is excreted mainly in the faeces, with urinary excretion representing a minor route. The ANS9801-acid metabolite has been detected in rat and human plasma. In contrast, in the dog there is little free ANS9801-acid in plasma. There was no evidence of accumulation of ANS9801-acid in healthy and type II diabetes subjects at either 4 or 12 weeks. This is consistent with the short estimated half-lives of radioactivity of ANS9801-acid from the preclinical and clinical studies. ANS9801-acid can be hydrolysed to phenylalanine, and an aspartic acid derivative, HF-1. HF-1 is a minor urinary metabolite in rats, dogs and humans. HF-1 can be further metabolised to HU-1, which is also postulated to be formed directly from ANS9801-acid. HU-1 is a minor urinary metabolite in rats, dogs and humans, which has not been isolated from the faeces of any species. In human and dogs, HF-1 was the major faecal metabolite, however in rats the demethylated ANS9801-acid was the major faecal metabolite. Other minor urinary and faecal metabolites have been detected but not characterised. Studies were only carried out with advantame radiolabelled on the 3-hydroxy-4-methoxy-phenyl moiety of the molecule, and whilst the metabolic fate of the remainder of the molecule could not been investigated, it could be predicted. The applicant concluded that the rat is a suitable metabolic model for systemic metabolites of advantame in human. They further concluded that effects due to differences in faecal metabolites between rats and human would have been addressed by the similarities in faecal metabolites between dogs and human. The Panel agreed that despite some minor differences in the metabolic studies, the experimental metabolic data support the use of the available toxicological studies in rat and dogs for the safety assessment of advantame in humans.

Considering toxicokinetic data derived from subchronic or chronic toxicity studies in mice, rats and dogs, the Panel noted that in all animal species the $C_{max}$ of advantame and ANS9801-acid were observed 0.5 to 1 hour post-dosing, indicating a rapid absorption and metabolism of advantame. The Panel also noted that in all studies, whatever the dose was, $AUC_{24}$ of ANS9801-acid were 5 to 300-fold higher than those of advantame, depending on animal species. This indicates an extensive metabolism of advantame to its metabolite, ANS9801-acid.

The Panel noted that in 13-week dietary studies in mice and rats, in a chronic toxicity and carcinogenicity study in rats and in a subchronic toxicity study in dogs, the rate and extent of systemic exposure of animals to advantame and ANS-9801 acid were highly variable and appeared to be characterised by non-linear kinetics and with no clear underlying mechanism over the dietary

concentration range used in these experiments. By considering the changes in $AUC_{24}$ data in the course of these subchronic or chronic studies, the Panel considered that advantame or ANS9801-acid do not accumulate even in case of high dietary doses given to these animal species for periods as long as 52 or 104 weeks.

Toxicokinetic data derived from a prenatal developmental toxicity study in rabbits demonstrated that when advantame administered by gavage at 2000 mg/kg bw/day, a dose similar to that achieved at advantame dietary concentration of 50 000 mg/kg diet in subchronic toxicity study in dogs and chronic toxicity and carcinogenicity study in rats, the systemic exposure to both advantame and ANS9801-acid is 5 to 25 times higher than in dietary administered animals. This would represent a worst case situation for a maximal systemic exposure of animals to advantame and ANS9801-acid. The Panel also noted that in this study, advantame and ANS-9801-acid were described to accumulate at the higher doses since increasing the dose of advantame resulted in a disproportionately higher systemic exposure to advantame than would be predicted from a linear relationship.

Overall, the Panel considered that the high variability and the lack of clear underlying mechanism did not permit such a clear conclusion on accumulation or non-linearity in kinetics.

The safety of advantame has been examined in *in vitro* studies, in sub-acute, subchronic and long-term *in vivo* studies in mice, rats and dogs and in reproductive and developmental studies in rats and rabbits. All studies were performed according to OECD guidelines and were conducted under conditions of GLP.

There is no concern with respect to the acute toxicity of advantame based on the data available from the oral route exposure study in rats ($LD_{50}$ for advantame in rats is > 5000 mg/kg bw).

In all of the subchronic toxicity studies apart from the 13-week study in rats and 1-year study in dogs decreases in body weight/body weight gain were seen at a dietary concentration of 50 000 mg advantame/kg diet. In the 13-week study in mice the difference in body weight gain did not attain a statistical significance. The statistically significantly lower body weight and the body weight gain were recorded in both sexes in the 4-week rat study (the body weight gains were 82 to 85 % of the control for males and females, respectively) while in dog studies the statistical significance was recorded in one sex only (for females in the 4-week study the body weight gain was 58 % of the control; for males in the 13-week study the body weight gain was 30 % of the control). In these studies food consumption in treated groups was comparable to the controls but decreases in food efficiency were recorded.

In the subchronic studies several parameters of the laboratory examinations were statistically significantly altered at a dose which affected body weight/body weight gain or lower. The recorded changes, although statistically significant, were often slight and within the physiological ranges for the species (Evans, 2009a,b), the dose-response was not apparent, they were not accompanied by morphological changes in any organs and there was no consistency between the sexes in the recorded changes, or between the studies in the same species or between the species. For instance, decreased haemoglobin and haematocrit in males, and leucocyte and lymphocyte counts in females were recorded in the 4-week rat study at advantame dietary concentrations of 1500 mg/kg diet and above. In the 13-week study in rats haemoglobin and haematocrit were significantly decreased at 15 000 mg/kg diet only in females and in both sexes at 50 000 mg/kg diet. In the dog haematocrit and haemoglobin were unremarkable in the 4-week study, were decreased in the 13-week study at 15 000 mg advantame/kg diet and above, but were increased in the 1-year study (haematocrit at 10 000 mg/kg diet and above; haemoglobin at 2000 mg/kg diet and above). In the dog, lymphocyte counts were increased in the 4-week study at 15 000 mg advantame/kg diet and above but were not statistically significantly different from the controls in the 13-week and 1-year study; leucocyte counts were only statistically significantly lower in the 13-week study. The Panel considered that the changes in laboratory parameters seen in the subchronic studies could be considered random.

The Panel noted, however, that the decreases in leucocyte and lymphocyte numbers in the subchronic studies correlated with changes in several parameters indicative of an altered function of the immune system in the 13-week study in rats (lower lymphocytes mitogenic responsiveness to Concanavalin A, lower total T-cells, lower CD4+, higher number of CD8+ cells) and with the observation of lower relative thymus weight in the 4-week rat study, and in the 4-week and 13-week (not statistically significant) dog studies in one of the sexes. The results from subchronic studies with advantame in rodents and dogs were equivocal as regards immunotoxicity and, therefore, the Panel required additional studies in young rats investigating the effects of advantame on various immunological parameters following short exposure and with groups including a post-dosing recovery phase to dose levels of 15 000 mg/kg diet and lower. An additional study (TNO Triskelion report, 2013), investigating the potential immunotoxic effects of dietary administered advantame in young male and female Wistar rats, was submitted to EFSA. There were no treatment-related differences in total or differential white blood cell counts or in total protein concentration, albumin concentration and albumin/globulin ratio. The weights of the adrenals, spleen and thymus at the end of treatment and the recovery period were not affected by treatment. Macroscopic examination and microscopic examination of lymphoid organs at the end of the treatment did not reveal any treatment related findings. There were no treatment-related differences in lymphocyte subsets: T cells (CD3+), Helper T cells (CD3+ CD4+), cytotoxic T cells (CD3+ CD8a+), B cells (CD45RA+), lymphocytes (CD45+), NK cells (NKR-P1A+). No treatment-related effects on proliferative response of splenocytes to mitogen stimulation by Con A were observed. There were no treatment-related differences in any cytokine production after mitogen stimulation. Because no treatment-related changes were observed in the main groups, the above determinations were not conducted in the recovery groups. Overall, the Panel considered that this 4-week study did not reveal any immunotoxic effects of advantame on the endpoints that were examined in the rat; the NOAEL from this study was 1380 mg advantame/kg bw/day. This study was conducted according to GLP and included evaluation of several key immune functions in the young animal. Under the conditions of this 4-week study, the Panel noted that the data provided evidence for an absence of immunotoxic effects of advantame as were suggested by the previous rat 13-week study.

Advantame was found to be not genotoxic in the bacterial reverse mutation assay, the *in vitro* mouse lymphoma TK assay and the *in vivo* mouse micronucleus assay.

In the carcinogenicity study in mice malignant tumours with a statistically significantly increased incidence in some treated groups were bronchio-alveolar adenocarcinoma (low dose females, $p < 0.05$) and histiocytic sarcoma of the haematopoietic system (low dose males, $p < 0.05$). The pulmonary tumours are known to have a high background incidence in mice. For both tumours there was no dose-response relationship in both sexes and the incidences were within historical control ranges for 78-104-week old CD-1 mice. Thus the Panel considered that these malignant tumours were not treatment-related. Advantame was considered by the Panel not to be carcinogenic to mice at doses up to 50 000 mg/kg diet, the highest concentration tested, as recommended in the OECD Guideline 451, and equal to 5693 and 7351 mg/kg bw/day in males and females, respectively. The Panel noted that at the high dose the body weight gain was significantly lower than controls in females (76 % of control) and a trend toward lower body weight was also observed for male mice, however the difference did not achieve statistical significance. With regard to the lower body weight gain in the high-dose group the NOAEL was 10 000 mg advantame/kg diet, equal to 1057 and 1343 mg advantame/kg bw/day in males and females, respectively.

In the carcinogenicity study in rats a higher incidence of pancreatic islet-cell carcinoma or of pancreatic islet-cell carcinoma and adenoma combined was seen in high-dose males but the incidence was not statistically significantly different from that in controls and these pancreatic tumour incidences were within the historical control values. The incidence of mammary gland adenoma in high-dose females was statistically significantly higher than that of controls but it was within the range of the historical control values. In contrast the incidence of mammary adenocarcinoma was not statistically increased in any of the treated groups. Combining the incidence of mammary gland adenomas and adenocarcinomas no pairwise comparisons achieved statistical significance. When the

authors of the study combined the incidence of mammary gland adenomas, adenocarcinoma and fibroadenoma a positive trend was noted with dose but no pairwise comparisons achieved statistical significance. The Panel noted that while combining the incidence of adenoma and adenocarcinoma is a common practice, helpful in considerations of a carcinogenic effect of a test compound, as both tumours originate from the same cellular structures, combining incidence of fibroadenoma with those of adenoma and adenocarcinoma is not a common practice and provides little information. Since the incidence rates of all mammary tumour types and combinations were within the historical control range, and as mammary tumours are part of the background pathology of the aging female rat, the Panel considered the increased incidences of mammary tumours in this study to be unrelated to treatment. Consequently the Panel considered that there was no evidence of carcinogenicity in rats treated with advantame in doses corresponding to concentrations of 50 000 mg/kg diet, the highest dose tested. This dietary concentration was equal to 2621 or 3454 mg advantame/kg bw/day in male and female rats, respectively. The Panel noted that at the high dose body weight gains in the males (93 % of control) and females (94 % of controls) treated for 104 weeks were reduced, attaining statistical significance in males only.

Reproductive and developmental toxicity studies on advantame included a 2-generation reproduction toxicity study in rats, and prenatal developmental studies in rats and rabbits. The NOAEL for reproductive toxicity in 2-generation reproduction toxicity study in rats was 50 000 mg advantame/kg diet, the highest dose tested. The NOAEL in rats for maternal toxicity from the prenatal developmental study was 15 000 mg advantame/kg diet (equal to 1419 mg/kg bw/day) and for developmental effects was 50 000 mg advantame/kg diet/day (equal to 4828 mg/kg bw/day) the highest dose tested. In rabbits the NOAEL for maternal toxicity was 500 mg advantame/kg bw/day based on disturbances of gastrointestinal tract causing morbidity that required killing the animals for welfare reasons at the next highest dose (1000 mg/kg bw/day) and the NOAEL for developmental toxicity was 1000 mg advantame/kg bw/day.

In other special studies conducted in rats, no behavioural or physiological alterations were observed and no mortality resulted from the administration of advantame. In addition, the locomotor activity of rats was unaffected by the administration of advantame compared with vehicle-treated animals. Although no significant effects were reported on the gastrointestinal motility of rats treated with 10 or 100 mg/kg bw of advantame a dose of 1000 mg/kg bw resulted in a moderate statistically significant decrease in gastrointestinal motility compared to rats administered the vehicle. However, studies in dogs showed that the duodenal administration of advantame resulted in no significant effects on general respiratory status, resistance of the peripheral vasculature, and on electrical status of the myocardium compared with the vehicle control group. Intra-duodenal administration of advantame produced no biologically meaningful effects on cardio-respiratory parameters.

The human tolerability of advantame has been tested in three clinical studies (a single-dose study in healthy volunteers, a 4-week study in healthy volunteers, and a 12-week study in normo-glycemic and diabetic subjects). The results of the clinical trials demonstrated that advantame was well tolerated in single or repeated doses up to 0.5 mg/kg bw, in healthy volunteers and in both normo-glycemic individuals and diabetics. There were no serious adverse events or withdrawals during the study periods. The majority of adverse events were reported to be mild and unlikely to be related to the consumption of advantame; however, the development of pruritis in a normo-glycemic male subject in the advantame group was considered to be possibly related to treatment. A small number of adverse events were reported in diabetic subjects and reported to occur in equal frequency in both the treatment and control groups. One diabetic subject experienced three adverse events that were of moderate severity and were considered to be possibly related to treatment; however, all were resolved prior to the end of the study. No significant changes in vital signs (e.g., systolic and diastolic blood pressure, or pulse rate) were reported and advantame did not affect the various biochemical, haematological, or urinalysis parameters measured, plasma levels of glucose or insulin in normo-glycemic individuals, or alter glucose tolerance or insulin resistance in the diabetic subjects.

The applicant also considered potential toxicity of the major and minor degradation products of advantame but did not conduct any toxicological test in support of the safety of ANS9801-acid, the major degradation product of advantame, stating that the series of toxicological studies conducted to assess the safety of advantame also can be used to support the safety of ANS9801-acid. The Panel concurred with this view, based on its evaluation of the available metabolism and toxicokinetic data and that systemic exposure to ANS9801-acid was demonstrated in subchronic and long-term studies by results of measurement of this metabolite in the plasma of the treated animals. β-ANS9801, β-ANS9801-acid, ANS9801-imide, HF-1, and L-phenylalanine methyl ester were identified as the minor degradation products of advantame. L-Phenylalanine methyl ester is expected to hydrolyse to L-phenylalanine and methanol; therefore further studies were not conducted to assess its safety. Since β-ANS9801, β-ANS9801-acid, ANS9801-imide, and HF-1 have not been identified as impurities of the final advantame product, these degradation products were assessed individually in a series of *in vitro* genotoxicity assays. ANS9801-imide was also tested *in vivo* in a mouse micronucleus assay. Considering the results of these studies, as well as the low levels of dietary exposure, the applicant concluded that the minor degradation products of advantame were not expected to be associated with any adverse effects following oral consumption of advantame by humans under the proposed conditions of use. The Panel concurred with this conclusion. The Panel has reviewed data on phenylalanine and methanol in an ongoing evaluation of another sweetener which also produces those metabolites. On that evaluation, the Panel had considered that levels of phenylalanine and methanol much higher than those that could be released from advantame would not be of safety concern.[10]

Since advantame is a secondary amine, the applicant evaluated the potential to participate in the formation of *N*-nitroso compounds in foods and beverages and in the stomach. No nitrosation of advantame was identified by the applicant and, therefore, the Panel considered that the potential formation of *N*-nitroso advantame does not raise a safety concern.

After consideration of all the data on advantame and its metabolites and major and minor degradation products available for this evaluation, the Panel concluded that there were sufficient data with which to establish an ADI.

There is adequate evidence both for advantame and its metabolites and degradation products, that there are no concerns about genotoxicity, carcinogenicity and reproductive toxicity.

In the light of clarified concern for possible immunological effects (as discussed above) the critical studies which need to be considered in setting the ADI are those indicating body weight effects in the laboratory animals and studies on developmental toxicity with regard to maternal toxicity and gastrointestinal symptoms in the rabbit. Each of these will be considered in turn.

Decrease in body weight/body weight gain after feeding laboratory animals with high doses of intense sweeteners is a phenomenon associated with poor palatability and/or lower nutritional value of the diets containing high concentrations of the test material (Chowaniec and Hicks, 1979; Grice and Goldsmith, 2000; Flamm et al., 2003; Mahew et al., 2003). The lower body weight/body weight gain of variable severity were seen at dietary concentration of 50 000 mg advantame/kg diet in some of the subchronic toxicity studies (apart from 13-week study in rats and 1-year study in dogs), in the carcinogenicity study in mice and in the combined chronic toxicity and carcinogenicity study in rats. In these studies the food consumption was either similar to that of the controls or subject to a transitory increase (i.e. in the 13-week study in mice, 104-week study in mice and 52- and 104- week studies in rats). In view of the lack of overt toxicity in these studies the Panel considered that the effect on body weight/body weight gain could be attributed to lower nutritional value of the diets containing high concentrations of the non-nutritive test material. Therefore this effect, although treatment-related, was not considered a pivotal effect on which to base an ADI. In contrast, in the prenatal

[10] Draft scientific opinion on the re-evaluation of aspartame (E 951) as a food additive. Public consultation. Panel on Food Additives and Nutrient Sources added to Food (ANS). Available online: http://www.efsa.europa.eu/en/consultationsclosed/call/130108.pdf

developmental study in rats, the lower maternal body weight gain of animals exposed to 50 000 mg advantame/kg diet was observed in presence of a concomitant decrease in feed intake. The Panel considered both effects unlikely due to poor palatability of the feed with a high concentration of advantame as food consumption was not decreased in other studies in rats fed the same dietary concentration of the sweetener. Therefore the lower body weight gain of the dams at this dietary dose was considered as maternal toxicity.

In the prenatal developmental toxicity study in NZW rabbits by oral administration by gavage a dose-related effect on bodyweight gain occurred during the first 4 days of treatment, however the effect was not dose-related thereafter. No statistically significant effects were observed upon fetal examination regarding major abnormalities and their incidences or on abortion rates reported. Five of the 24 (21 %) mated females rabbits from the 2000 mg/kg bw/day group, sacrificed prematurely (between days 17 - 27) for animal welfare reasons, manifested high bodyweight loss (from 200 to 570 g) and a range of clinical signs and necropsy findings that suggested compound related toxicity. In addition to general staining of different body fluids and tissues, the animals showed reduced body temperature, decreased water intake and vocalization and overactive behaviour suggesting acute toxicity. Upon necropsy consistent findings such as large amounts of air and stained fluids and material in the caecum, in the GI tract and in the stomach as well as few soft faecal pellet formation in the rectum were reported. In some animals, either pale liver or liver with a pronounced lobular pattern, an enlarged gall bladder, multiple cysts in both kidneys and covering of the entire wall of the caecum with haemorrhagic gelatinous material were diagnosed. In the 1000 mg/kg bw/day group one animal out of twenty-four (5 %) had to be prematurely sacrificed and presented similar findings as those previously reported at the highest dose: high weight loss (710 g), decreased water intake, staining of fluids and tissues, loose faeces and stained faeces, difficulty to breath, vocalization, loss of locomotion and coordination. Necropsy examination showed additionally to the staining of some organs, kidneys with multiple punctuate foci on the surfaces, caecum and stomach walls covered with gelatinous material, caecum wall haemorrhagic and part of the stomach walls adjacent to the pyloric sphincter thickened. In the control group two animals out of twenty-four (10 %) were prematurely sacrificed during the study, however although one of those animals showed also decrease water intake, convulsion, difficulty to breath and vocalization, upon necropsy examination none showed the same effects in the gastrointestinal tract as those reported in the treated groups.

The Panel considered the findings in the prematurely sacrificed rabbits from the prenatal developmental study to be related to advantame exposure. The Panel noted that although only one animal was affected in the mid-dose group (1000 mg/kg bw/day), the effects observed in this animal were consistent with those found in sacrificed animals from the higher dose group (2000 mg/kg bw/day). The Panel considered that the single incidence of adverse effects in the mid-dose group may be indicative of the proximity of the boundary dose for adverse effects of advantame, since incidence increased in the higher dose group. Taking into account that effects indicative of maternal toxicity in form of decreased feed intake–were also observed in the developmental toxicity study in rats upon administration of advantame through the diet, the Panel considered that effects observed in the rabbits cannot be disregarded. Therefore the Panel identified a NOAEL of 500 mg advantame/kg bw/day for maternal toxicity in the prenatal developmental toxicity study in NZW rabbits by oral gavage.

This NOAEL was further supported by the difference in the toxicokinetics of high doses of advantame (around 2000 mg/kg bw/day) in the subchronic and chronic dietary administration studies in mice, rats and dogs and the oral gavage administration in reproductive and prenatal developmental toxicity studies in the rabbit. The Panel noted that the internal doses of advantame and ANS9801-acid as estimated by $AUC_{24}$ were markedly higher following oral gavage administration in the rabbit compared to dietary administration in rat, mouse and dog. The Panel noted that at low doses the kinetics following oral bolus administration in laboratory rodents and dogs and humans were similar. However many of these studies reported radioactivity rather than individual compound kinetics and as such the results from these studies were not directly comparable to the high dose kinetic results. Further the Panel noted that the kinetic parameters appeared linear at low doses. There were no high dose kinetic studies by gavage available for rat, mouse and dog nor were high dose dietary kinetic data

available for the rabbit. Therefore the Panel was unable to ascertain whether the observed differences in internal dose were due to the route of administration or were species specific. However given the similarity of kinetics at low bolus doses in all species studied including humans, the Panel considered that it would not be appropriate on the available information to disregard the oral gavage study because of the apparent increase in internal dose. Therefore, the Panel considered that the NOAEL of 500 mg advantame/kg bw/day for maternal toxicity from the rabbit developmental toxicity study should be used as the point of departure for the derivation of the ADI.

The Panel noted that either a default uncertainty factor of 100 or a lower uncertainty factor based on inter-species allometric scaling[11] and inter-human variability could be applied.. However, the Panel considered that the more conservative of these uncertainty factors would be appropriate in this case due to the marked variability in the internal doses arising following dietary or bolus administration which was seen at both high and low dose levels, the absence of complete kinetic data set (high and low dose with both methods of administration) in any species studied and the corresponding difficulty in defining a clear kinetic model. Therefore, the Panel considered that a 100-fold uncertainty factor for possible inter-species and inter-human variability should be used in this case. Applying this uncertainty factor of 100 to the NOAEL of 500 mg advantame/kg bw/day for maternal toxicity from the rabbit prenatal developmental toxicity study the Panel derived an ADI of 5 mg/kg bw/day.

Anticipated exposures to advantame from its proposed use as a food additive and its metabolites (methanol and phenylalanine) have been calculated (mean and 95$^{th}$ percentile of consumers only) using the food consumption data at the individual level (e.g. raw data on food consumption by the individual consumer).

High-level consumption was only calculated for those foods and population groups where the sample size was sufficiently large to allow calculation of the 95$^{th}$ percentile. The Panel estimated chronic exposure for the following population groups: toddlers, children, adolescents, adults and the elderly. Calculations were performed using individual body weights.

Thus, for the present assessment, food consumption data were available from 26 different dietary surveys carried out in 17 different European countries, as mentioned in Table 3.

When considering the proposed maximum use levels, the mean dietary exposure to advantame in European children-adolescents (aged 1-17 years) ranged from 0.02 to 0.33 mg/kg bw/day, and from 0.05 to 0.74 mg/kg bw/day for high level consumers (95$^{th}$ percentile). Exposure estimates for the European adult population give a mean dietary exposure to advantame ranged from 0.01-0.12 mg/kg bw/day, and from 0.03-0.28 mg/kg bw/day for high level consumers (95$^{th}$ percentile).

The Panel noted that its estimates could be considered as being conservative as it was assumed that all processed foods and beverages contained the sweetener advantame added at the maximum proposed use levels. It has to be noted that anticipated exposure to advantame did not include possible applications from its use as an excipient in pharmaceutical products, an application which might occur if advantame were an approved food additive

The exposure to methanol, which may result from ingestion of advantame-containing foods and beverages, is considered negligible compared to that from other dietary sources and as such of no concern from the safety point of view.

The exposure to phenylalanine expected from ingestion of advantame as a general purpose sweetener is considered of no safety concern for healthy consumers (adults and children). For a phenylketonuric child, the additional phenylalanine intake expected from all food and beverage applications of the sweetener will represent a relatively small increment in the exposure to phenylalanine.

[11] Guidance on information requirements and chemical safety assessment. Chapter R.8: Characterisation of dose [concentration]-response for human health. Version 2. December 2010.ECHA. Available online http://echa.europa.eu/documents/10162/13632/information_requirements_r8_en.pdf

## CONCLUSIONS

The Panel reviewed an extensive set of *in vitro* and *in vivo* animal studies and available human tolerance studies on advantame. After considering all the data available, the Panel concluded that advantame does not raise concern with regards to genotoxicity and carcinogenicity. The critical effect observed in animal studies was maternal toxicity (gastrointestinal disturbances) in the prenatal developmental toxicity study in rabbits. The NOAEL for this effect was 500 mg advantame/kg bw/day.

Studies in humans demonstrated that daily doses of advantame up to 0.5 mg/kg bw were well tolerated by individuals with normal glucose metabolism or type II diabetes.

The Panel considers that the information available is sufficient to conclude that any possible nitrosation of advantame, should it occur, is not a matter of concern.

The Panel, established an ADI of 5 mg/kg bw/day based on the application of a 100-fold uncertainty factor to the NOAEL of 500 mg advantame/kg bw/day for maternal toxicity from the prenatal developmental toxicity study in the rabbit.

Conservative estimates of advantame exposure for high level adults and children consumers were below the ADI for the proposed use levels.

After considering all the data on stability, degradation products, toxicology and exposure, the Panel concluded that advantame would not be of safety concern at the proposed uses and use levels as a sweetener.

The Panel considered that a maximum limit for palladium and for platinum should be included in the specifications.

## DOCUMENTATION PROVIDED TO EFSA

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2. Additional data requested: AJO/183 (2005). ANS9801: Preliminary embryo-fetal toxicity study in the rabbit by oral gavage administration. Second amended final report. Submitted by Ajinomoto Co. Inc.on May 2011.

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## ANNEX A.

**Reports of biological and toxicological studies with advantame submitted in the application dossier**:

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AJO/154 (2001). ANS9801: Bacterial Mutation Assay.

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## ANNEX B.

**Summary of total estimated exposure to advantame (using the proposed use levels) per age class and survey*: mean and high level (mg/kg bw/day)**

| | Proposed use levels | |
|---|---|---|
| | Mean | High level |
| **Toddlers** | | |
| Belgium (Regional_Flanders) | 0.330 | |
| Bulgaria (NUTRICHILD) | 0.065 | 0.236 |
| Germany (DONALD_2006_2008) | 0.086 | 0.263 |
| Spain (enKid) | 0.165 | |
| Finland (DIPP) | 0.122 | 0.415 |
| Italy (INRAN_SCAI_2005_06) | 0.079 | |
| Netherlands (VCP_kids) | 0.284 | 0.737 |
| **Children** | | |
| Belgium (Regional_Flanders) | 0.231 | 0.549 |
| Bulgaria (NUTRICHILD) | 0.058 | 0.186 |
| Czech Republic (SISP04) | 0.104 | 0.243 |
| Germany (DONALD_2006_2008) | 0.129 | 0.298 |
| Denmark (Danish_Dietary_Survey) | 0.078 | 0.190 |
| Spain (enKid) | 0.119 | 0.342 |
| Spain (NUT_INK05) | 0.110 | 0.290 |
| Finland (DIPP) | 0.138 | 0.327 |
| Finland (STRIP) | 0.108 | 0.234 |
| France (INCA2) | 0.113 | 0.254 |
| Greece (Regional_Crete) | 0.055 | 0.161 |
| Italy (INRAN_SCAI_2005_06) | 0.046 | 0.141 |
| Latvia (EFSA_TEST) | 0.070 | 0.175 |
| Netherlands (VCP_kids) | 0.248 | 0.611 |
| Sweden (NFA) | 0.139 | 0.321 |
| **Adolescents** | | |
| Belgium (Diet_National_2004) | 0.053 | 0.143 |
| Cyprus (Childhealth) | 0.016 | 0.045 |
| Czech Republic (SISP04) | 0.053 | 0.137 |
| Germany (National_Nutrition_Survey_II) | 0.075 | 0.229 |
| Denmark (Danish_Dietary_Survey) | 0.046 | 0.123 |
| Spain (AESAN_FIAB) | 0.023 | 0.061 |
| Spain (enKid) | 0.051 | 0.135 |
| Spain (NUT_INK05) | 0.064 | 0.167 |
| France (INCA2) | 0.054 | 0.131 |
| Italy (INRAN_SCAI_2005_06) | 0.023 | 0.076 |
| Latvia (EFSA_TEST) | 0.045 | 0.112 |
| Sweden (NFA) | 0.070 | 0.189 |

| | Proposed use levels | |
|---|---|---|
| | Mean | High level |
| **Adults** | | |
| Belgium (Diet_National_2004) | 0.048 | 0.134 |
| Czech Republic (SISP04) | 0.022 | 0.068 |
| Germany (National_Nutrition_Survey_II) | 0.056 | 0.173 |
| Denmark (Danish_Dietary_Survey) | 0.025 | 0.079 |
| Spain (AESAN) | 0.026 | 0.085 |
| Spain (AESAN_FIAB) | 0.015 | 0.050 |
| Finland (FINDIET_2007) | 0.041 | 0.126 |
| France (INCA2) | 0.036 | 0.095 |
| United Kingdom (NDNS) | 0.042 | 0.124 |
| Hungary (National_Repr_Surv) | 0.017 | 0.062 |
| Ireland (NSIFCS) | 0.032 | 0.098 |
| Italy (INRAN_SCAI_2005_06) | 0.012 | 0.048 |
| Latvia (EFSA_TEST) | 0.024 | 0.061 |
| Netherlands (DNFCS_2003) | 0.117 | 0.280 |
| Sweden (Riksmaten_1997_98) | 0.045 | 0.118 |
| **The elderly** | | |
| Belgium (Diet_National_2004) | 0.036 | 0.102 |
| Germany (National_Nutrition_Survey_II) | 0.034 | 0.098 |
| Denmark (Danish_Dietary_Survey) | 0.017 | 0.056 |
| Finland (FINDIET_2007) | 0.019 | 0.071 |
| France (INCA2) | 0.024 | 0.067 |
| Hungary (National_Repr_Surv) | 0.010 | 0.030 |
| Italy (INRAN_SCAI_2005_06) | 0.008 | 0.038 |

* The different methodologies of European dietary surveys included in the EFSA Comprehensive Database are fully described in the Guidance on the use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment (EFSA, 2011a). A summary is available p.11, Table 1 of the guidance.

## ABBREVIATIONS

| | |
|---|---|
| ADI | Acceptable daily intake |
| ADME | Absorption, distribution, metabolism, excretion |
| AFSSA | Agence française de sécurité sanitaire des aliments |
| ALAT | Alanine aminotrasferase |
| ALKP | Alkaline phosphatase |
| ANS | Scientific Panel on Food Additives and Nutrient Sources added to Food |
| ANS9801 | Advantame |
| ANS9801-acid | *N*-[*N*-[3-(3-hydroxy-4-methoxyphenyl)propyl]-α-aspartyl]-L-phenylalanine |
| AUC | The area under the mean plasma concentration versus time curve |
| $AUC_{24}$ | The area under the mean plasma concentration-time curve estimated up to 24 hours post-dose |
| CAS | Chemical abstracts service |
| $C_{max}$ | Maximum mean plasma concentration |
| EAR | Estimated Average Requirement |
| EC | European Commission |
| ECG | Electrocardiography |
| EFSA | European Food Safety Authority |
| EU | European Union |
| FDA | Food and Drugs Administration |
| FOB | Functional observation battery |
| FSANZ | Food Standards Australia New Zealand |
| GD | Gestation day |
| GLP | Good laboratory practice |
| HPLC | High-performance liquid chromatography |
| HF-1 | *N*-(3-(3-hydroxy-4-methoxyphenyl))-propyl-L-aspartic acid |
| HMCA | 3-hydroxy-4-methoxycinnamaldehyde |
| HMPA | 3-(3-hydroxy-4-methoxyphenyl)propionaldehyde |

| | |
|---|---|
| HU-1 | 3-(3-hydroxy-4-methoxyphenyl)-1-propylamine |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |
| IOM | Institute of Medicine |
| IUPAC | International Union of Pure and Applied Chemistry |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| $LD_{50}$ | Lethal dose, 50 % i.e. dose that causes death among 50 % of treated animals |
| LOQ | Limit of quantification |
| NK | Natural killer |
| NOAEL | No observed adverse effect level |
| OECD | Organisation for Economic Co-operation and Development |
| RF-1 | *N*-[*N*-[3-(3,4-dihydroxyphenyl)propyl]-α-aspartyl]-L-phenylalanine |
| RH | Room humidity |
| SPE | Cation-exchange solid phase extraction |
| TNO | Nederlandse Organisatie voor toegepast-natuurwetenschappelijk onderzoek |
| UTI | Urinary tract infection |
| UV | Ultraviolet |
| WBC | White blood cells |