Adopted: 20 May 2025

DOI: 10.2903/j.efsa.2025.9480

efsa JOURNAL

SCIENTIFIC OPINION

Re-evaluation of neotame (E 961) as food additive

EFSA Panel on Food Additives and Flavourings (FAF) | Laurence Castle | Monica Andreassen | Gabriele Aquilina | Maria Lourdes Bastos | Polly Boon | Biagio Fallico | Reginald FitzGerald | Maria Jose Frutos Fernandez | Bettina Grasl-Kraupp | Ursula Gundert-Remy | Rainer Gürtler | Eric Houdeau | Marcin Kurek | Henriqueta Louro | Patricia Morales | Sabina Passamonti | Monika Batke | Ellen Bruzell | James Chipman | Riccardo Crebelli | Cristina Fortes | Peter Fürst | Eric Gaffet | Cheyns Karlien | Thorhallur Halldorsson | Jean-Charles Leblanc | Oliver Lindtner | Katrin Loeschner | Jan Mast | Manuela Mirat | Alicja Mortensen | Anna Undas | Matthew Wright | Stefania Barmaz | Consuelo Civitella | Jose Cortiñas Abrahantes | Pauline Le Gall | Elena Mazzoli | Agnieszka Mech | Josef Daniel Rasinger | Ana Rincon | Francesca Riolo | Camilla Smeraldi | Alexandra Tard | Panagiota Zakidou | Federica Lodi

Correspondence: fip@efsa.europa.eu

The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts

Abstract

The present opinion deals with the re-evaluation of neotame (E 961) as a food additive. Neotame is the chemically manufactured compound N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester. The main impurity of neotame (E 961) is also a degradation product (de-esterified form), N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751) and the primary metabolite. No new data were received following the call for biological and toxicological data. A summary of the toxicological studies available in the EFSA opinion of 2007 is presented and studies gathered from the literature are summarised. Neotame is rapidly absorbed and pre-systemically metabolised, systemic intact neotame is likely to be excreted in the urine with its metabolites. The potential aneugenic effects at the site of contact are not expected to occur; overall, there is no concern for genotoxicity of neotame (E 961) at the maximum permitted levels or reported use levels. A review of the other endpoints from the already available toxicological database did not indicate an adverse effect for neotame at the highest doses tested. The Panel established an acceptable daily intake (ADI) of 10 mg/kg bw per day for neotame based on the no observed adverse effect level (NOAEL) of 1000 mg/kg bw per day from a 52-week chronic and 104-week carcinogenicity studies in rats. This ADI replaces the ADI of 2 mg/kg bw per day established by EFSA in 2007. The resulting exposure to methanol and its metabolite formaldehyde from the use of neotame at the ADI of 10 mg/kg bw per day does not raise a concern. The dietary exposure estimates of neotame (E 961) for the different population groups of all exposure scenarios did not exceed the ADI. The Panel concluded that there is no safety concern for neotame (E 961) at the currently permitted and reported uses and use levels. The Panel recommended the European Commission to consider revising the EU specifications of neotame (E 961).

KEYWORDS

E961, food additive, neotame, re-evaluation, sweetener

CONTENTS

SUMMARY

The present opinion deals with the re-evaluation of neotame (E 961), authorised as a food additive in the European Union (EU) in accordance with Annex II and Annex III to Regulation (EC) No 1333/2008 on food additives.

Neotame was evaluated previously by JECFA (JECFA, 2004) and by the former Panel of EFSA on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (EFSA, 2007). Both committees established an ADI of 2 mg/kg bw per day based on the application of a 100-fold safety factor to the NOAEL of 200 mg/kg bw from a 52-week dog study.

Neotame is the chemically manufactured compound N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester obtained by reaction under hydrogen pressure of N-L-α-aspartyl-L-phenylalanine 1-methyl ester (aspartame) with 3,3-dimethylbutyraldehyde in methanol in presence of a palladium/carbon catalyst. This food additive is a product of chemical synthesis using well defined chemical precursors, involving several separation and purification steps. The analytical data provided for the purity of neotame (E 961) were higher than 98%. The Panel considered that the minimum percentage of neotame should be raised from 97% to at least 98% in the assay requirement outlined in the EU specifications.

The main impurity of neotame (E 961) is also a degradation product (de-esterified form) called N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751). The Panel noted that in the EU specifications, this impurity is referred to as N-[(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine and the name should be changed to N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine, to be consistent with the chemical name of the food additive itself. The Panel also noted that the major degradation product NC-00751 is also the primary metabolite. Therefore, the safety of this impurity is covered by the toxicity data available on neotame and does not give rise to concern as an impurity at the level of up to 1.5%.

The Panel noted that apart from NC-00751, the parameter 'other related substances' (Not more than 2.0%) as described in the specifications of JECFA (2003), is not included in the current EU specifications. The potential exposure to these 'other related substances', resulting from the use of E 961 was individually assessed using the threshold of toxicological concern (TTC) approach. The 'other related substances' (with the exception of L-phenylalanine and 3,3-dimethylbutylamine (Cramer Class I)) were classified into Cramer Class III (Appendix F). In order not to exceed the respective TTC value (1.5 μg/kg bw per day), their presence individually should be less than 0.9% in the food additive. The Panel noted that no concern is identified for these impurities and there is no need to include them in the EU specifications.

In addition, the Panel considered that information on its specific optical rotation should be included in the EU specifications.

The Panel considered that a microbiological contamination is unlikely and it is not necessary to recommend inclusion of microbiological criteria in the EU specifications for E 961.

Considering the results of the exposure to the toxic element lead (Pb), the Panel noted that its presence in E 961 at the current specification limit value would not give rise to concern, while for palladium (Pd) the Panel did not consider it necessary to propose a specification limit in the EU specifications for E 961. Furthermore, the Panel did not see a need to recommend additional specifications for arsenic (As), cadmium (Cd) and mercury (Hg).

The Panel noted that the current EU specifications include a solubility value of '*4.75% (w/w) at 60°C in water, and solubility in ethanol and ethyl acetate*'. According to the analytical data provided for the solubility of neotame (E 961), the Panel considered that neotame is 'sparingly soluble in water, very soluble in ethanol', in line with the JECFA specifications. The Panel suggests an amendment to the description of the solubility of E 961 in line with the JECFA specifications. In addition, the Panel took note of the consideration made by one IBO that 'ethyl acetate is not a particularly useful solvent for food applications, nor for analytical purposes' and concurred with the suggestion to remove the provision on solubility in ethyl acetate from the EU specifications of E 961.

Based on the submitted data, the Panel noted that the presence of small particles including nanoparticles in the food additive E 961 has been reported by the IBO under the examined conditions.

The reported solubility value (e.g. 12.6 g/L at 25°C) for neotame (E 961), is lower than the threshold value of 33.3 g/L, as a decision criterion for demonstrating that the material does not require specific assessment at the nanoscale (EFSA Scientific Committee, 2021b). The Panel noted that the maximum use levels of neotame (E 961) do not exceed 60 mg/kg and the highest MPL does not exceed 250 mg/kg (FC 5.3). Taking into account the maximum reported use levels, the MPLs, the reported solubility value, and the volume of gastric secretion (from 215 mL within a single meal to 2000 mL daily; ICRP, 2002; Mudie et al., 2014), the Panel considered that full dissolution of E 961, is to be expected in foods and/or in the gastrointestinal tract and that ingested particles (if any) would not persist. Therefore, the Panel considered that there is no concern with regards to the potential presence of small particles, including nanoparticles, in neotame (E 961) at the reported uses and use levels and the food additive can be assessed following the conventional risk assessment (EFSA ANS Panel, 2012).

The Panel noted that E 961 is stable in most food categories, but is prone to hydrolysis and other degradation pathways, particularly at very low pH and high temperature, to form mainly NC-00751, along with NC-00777, NC-00779 and NC00764, plus other minor degradation products.

The potential hazard related to nitrosation of neotame and its degradation products in the gastrointestinal tract was considered in the previous EFSA opinion (2007). The AFC Panel concluded that the hypothetical nitrosation of neotame was of no concern with respect to genotoxicity (EFSA, 2007). The FAF Panel agrees with this conclusion.

No new data were received following the call for biological and toxicological data. From the literature search performed, the new studies considered as relevant according to the inclusion criteria reported in the protocol on hazard identification and characterisation (EFSA, 2020a; EFSA FAF Panel, 2023), were summarised in this opinion.

In the present evaluation, a summary of the several toxicological studies in animal and human studies available at the time of the safety evaluation of neotame as a food additive (E 961) in 2007 (EFSA, 2007) is presented. In the case of neotame, the study on which the current ADI was based (i.e. the 52-week dog study) was considered of moderate risk of bias (RoB, Tier 2). According to the criteria outlined in the revised protocol (EFSA FAF Panel, 2023), with the key studies from the previous evaluation rated as tier 1 or tier 2 in the RoB assessment, the Panel considered it appropriate not to re-assess the other previously evaluated studies.

The FAF Panel considered that neotame is rapidly absorbed and pre-systemically metabolised in both animals and humans. Any systemic intact neotame is likely to be excreted in the urine along with its metabolites. A significant fraction of neotame metabolites may result from pre-systemic metabolism. The data are consistent with biliary excretion in dogs. The Panel noted that the major degradation product NC-00751 is also the primary metabolite of neotame, which implies that the available toxicological dataset also informs on the toxicological profile of NC-00751, being an impurity, a degradation product and a metabolite.

The results of in vitro genotoxicity assays indicate that neotame does not induce gene mutation. Considering the available experimental data, the Panel considered that the positive findings in the in vitro MN assay, in which neotame induced the formation of micronuclei in the presence and absence of metabolic activation, are due to aneugenicity. Considering the evidence of bioavailability of oral administered neotame, the Panel considered that genotoxic effects due to systemic exposure to neotame and its metabolites are not expected. However, the available data were insufficient to address the possible aneugenic effects of neotame at the site of contact (oesophagus and stomach).

In the absence of validated test methods to assess aneugenicity in the upper GIT, following the relevant EFSA Guidance (EFSA Scientific Committee, 2021a), the Panel compared the concentrations resulting in induction of micronuclei in vitro with the MPL for beverages (FC 14.1.3 and FC 14.1.4), i.e. assuming the highest concentration of neotame in oesophagus and stomach is equal to the MPL. To identify a reference point for aneugenicity assessment, the in vitro data from the micronucleus test were subjected to a benchmark dose (BMD) analysis using the EFSA 'Bayesian BMD' webtool. The FAF Panel decided to use historical negative control data for deriving the respective BMRs for the 4 and 24 h MN assays (without metabolic activation). Taking into account that the data used for this analysis were concentrations and not doses, the Panel considered it appropriate to use the term benchmark concentration (BMC), rather than BMD.

As a worst-case scenario, the Panel compared the BMCL of the 24 h MN assay to the concentration equal to the currently authorised maximum permitted levels (MPL) of 20 mg/L in FC 14.1.3 and FC 14.1.4 corresponding to beverages (Table 4). The Panel noted that this BMCL (156 mg/L) is approximately eight-fold higher than the MPL of 20 mg/L for neotame (E 961) in beverages. Comparing the reported use levels and analytical data in beverages (7 mg/L for FC 14.1.3 and 2.8 mg/L for FC 14.1.4, respectively) with the BMCL would result in a difference of more than an order of magnitude.

The Panel considered that aneugenic effects at the site of contact are not expected to occur under the intended condition of use of neotame. Furthermore, as recommended by the EFSA SC statement (EFSA Scientific Committee, 2017), other toxicological data were considered that may assist in supporting this consideration. There was no indication for carcinogenicity in two studies in mice and rats with doses up to 1000 mg/kg bw per day and no adverse effects in the GIT (Section 3.5.4) or other systemic adverse effects in additional studies, including three reproductive and developmental toxicity studies (Section 3.5.5).

Overall, the Panel considered that there is no concern for genotoxicity of neotame (E 961) at the MPL or reported use levels.

Concerning systemic toxicity, the FAF Panel considered that the increase in hepatic alkaline phosphatase activity in the serum in the dog studies (i.e. a 13-week- and a 52-week study on which the current ADI is based on) was not a marker of an adverse effect in the absence of other toxicological effect in the liver. The Panel noted that this conclusion is in line with ongoing discussion in the scientific literature (e.g. Yokoyama et al., 2019).

A review of the other endpoints in the available toxicological database did not indicate an adverse effect for neotame at the highest doses tested in rodents, i.e. up to doses of 3000 mg/kg bw per day in a 13-week study in rats, up to 1000 mg/kg bw per day in a 52-week chronic and 104-week carcinogenicity studies in rats, and up to 4000 mg/kg bw per day in mice dosed for 104 weeks. The Panel noted that at the highest doses in all studies there was reduced feed intake associated with decreased body weight gain, which were considered related to reduced palatability in rodents (Flamm et al., 2003; Mayhew et al., 2003).

In light of the above, a reference point (RP) of 1000 mg/kg bw per day from a 52-week chronic and 104-week carcinogenicity studies in rats is chosen to derive the ADI for neotame. However, the Panel considered that, in order to establish a health-based guidance value (HBGV) for neotame, the resulting exposure to methanol and its metabolite formaldehyde from the use of neotame should be taken into account to ensure that the dietary exposure to the food additive does not raise a concern.

Based on the RP of 1000 mg/kg bw per day, a five-fold higher ADI of 10 mg/kg bw per day would be derived. This would increase the steady state concentration of methanol and the peak level of formaldehyde. The exposure to formaldehyde was estimated using worst-case assumptions. The resulting steady state level and peak level of formaldehyde in humans were compared to data in rats on the concentration of formaldehyde in blood and the interindividual variation (Kleinnijenhuis et al., 2013). In the rat study, the coefficient of variation was 30% for the formaldehyde concentration. An increase of the ADI to 10 mg/kg bw per day would result in an increase of about 4% (steady state level) and 18.5% (peak level) of formaldehyde background, which is within the observed background variation of 30%.

The FAF Panel considered that the amount of phenylalanine which could be potentially formed from neotame (E 961) at the proposed ADI of 10 mg/kg bw per day would be safe for the general population, including individuals heterozygous

for the phenylalanine hydroxylase gene, as it would increase the physiological phenylalanine concentration by less than 1% (EFSA ANS Panel, 2013).

Based on these considerations, the Panel considered appropriate to establish an ADI for neotame (E 961) of 10 mg/kg bw per day based on application of a default 100-fold uncertainty factor to the NOAEL of 1000 mg/kg bw per day, the highest dose tested, from a 52-week chronic and 104-week carcinogenicity studies in rats. Accordingly, this ADI replaces the ADI of 2 mg/kg bw per day established by the EFSA AFC Panel in 2007.

Dietary exposure to neotame (E 961) was estimated according to different exposure scenarios based on consumers only. Currently, neotame (E 961) is an authorised food additive in the EU in 34 food categories, while concentration data were available for only 5 categories.

Neotame (E961) is not commonly used in Europe, as indicated by the Mintel GNPD and literature, therefore, the Panel considered the exposure to neotame (E 961) from its use as food additive, to be an overestimation in all scenarios.

The Panel considered *the refined brand-loyal exposure assessment scenario*, the most appropriate exposure scenario for the risk assessment. In this *scenario*, mean exposure to neotame (E 961) ranged from less than or equal to 0.01 mg/kg bw per day in all age groups to 0.05 mg/kg bw per day in infants and toddlers. The 95th percentile of exposure ranged from 0.01 mg/kg bw per day in adolescents, adults and the elderly to 0.16 mg/kg bw per day in toddlers.

The Panel noted that the dietary exposure estimates of neotame (E 961) at the mean and P95 in all population groups for all scenarios did not exceed the ADI of 10 mg/kg bw per day for neotame (E 961). Therefore, the Panel concluded that there is no safety concern for neotame (E 961) at the currently permitted and reported uses and use levels.

Uncertainties identified for exposure assessments and genotoxicity assessment were considered not to affect the overall conclusions regarding the safety of neotame (E 961).

The Panel recommends the European Commission to consider:

- changing the name of the impurity N-[(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine to N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine in the EU specifications;
- raising the minimum percentage of neotame from 97% to at least 98% in the assay requirement outlined in the EU specifications;
- introducing information on the specific optical rotation in the EU specifications of E 961; and
- revising the solubility parameter to 'sparingly soluble in water, very soluble in ethanol'.

1 | INTRODUCTION

The present opinion deals with the re-evaluation of neotame (E 961) when used as a food additive.

1.1 | Background and Terms of Reference as provided by the requestor

1.1.1 | Background

Regulation (EC) No 1333/2008[1] of the European Parliament and of the Council on food additives requires that food additives are subject to a safety evaluation by the European Food Safety Authority (EFSA) before they are permitted for use in the European Union (EU). In addition, it is foreseen that food additives must be kept under continuous observation and must be re-evaluated by EFSA.

For this purpose, a programme for the re-evaluation of food additives that were already permitted in the European Union before 20 January 2009 has been set up under the Regulation (EU) No 257/2010.[2] This Regulation also foresees that food additives are re-evaluated whenever necessary in the light of changing conditions of use and new scientific information. For efficiency and practical purposes, the re-evaluation should, as far as possible, be conducted by group of food additives according to the main functional class to which they belong.

The order of priorities for the re-evaluation of the currently approved food additives should be set on the basis of the following criteria: the time since the last evaluation of a food additive by the Scientific Committee on Food (SCF) or by EFSA, the availability of new scientific evidence, the extent of use of a food additive in food and the human exposure to the food additive taking also into account the outcome of the Report from the Commission on Dietary Food Additive Intake in the EU of 2001. The report "Food additives in Europe 2000" submitted by the Nordic Council of Ministers to the Commission, provides additional information for the prioritisation of additives for re-evaluation. As colours were among the first additives to be evaluated, these food additives should be re-evaluated with a highest priority.

In 2003, the Commission already requested EFSA to start a systematic re-evaluation of authorised food additives. However, as a result of adoption of Regulation (EU) 257/2010 the 2003 Terms of References are replaced by those below.

1.1.2 | Terms of Reference

The Commission asks the European Food Safety Authority to re-evaluate the safety of food additives already permitted in the Union before 2009 and to issue scientific opinions on these additives, taking especially into account the priorities, procedures and deadlines that are enshrined in the Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with the Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives.

1.2 | Information on existing authorisations and evaluations

Neotame (E 961) is authorised as a food additive in the EU in accordance with Annex II to Regulation (EC) No 1333/2008 on food additives and its specifications are defined in the Commission Regulation (EU) No 231/2012.[3]

Neotame was previously evaluated by JECFA in 2003 when an Acceptable Daily Intake of 2 mg/kg bw per day was set (JECFA, 2004). According to the JECFA evaluation: '*the only consistent treatment-related effect observed was an increase in serum alkaline phosphatase activity in the 13-week and 1-year studies in dogs fed neotame in the diet. While the increase in alkaline phosphatase was moderate, reversible, and was not accompanied by other evidence of liver toxicity, the observed change was reproducible, of high statistical significance and treatment-related. The Committee agreed there were insufficient data to discount this effect and therefore accepted the dog as the most sensitive species with a NOEL for neotame of 200 mg/kg of body weight per day, on the basis of the 1-year study in dogs fed neotame in the diet. Studies of toleration in humans confirmed the lack of any treatment-related signs or symptoms at doses of up to 1.5 mg neotame/kg of body weight per day in diabetic and nondiabetic subjects. Although a 1-year study is not considered to be a long-term study in dogs, an additional safety factor was not considered necessary, in light of the human data*' (JECFA, 2004).

Neotame (E 961) was previously evaluated by the former EFSA Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC), for its authorisation for use as a new food additive (EFSA, 2007). The conclusions by the AFC Panel were based on the same critical endpoint (increase in serum alkaline phosphatase) and the same no observed adverse effect level (NOAEL) (i.e. 200 mg/kg bw per day) from a 52-week study in dogs (PCR 1017 as cited in the

[1]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. 16.

[2]Commission Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re- evaluation of approved food additives in accordance with Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives. OJ L 80, 26.3.2010, p. 19.

[3]Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) no 1333/2008 of the European Parliament and of the Council. OJ L 83, 22.3.2012, p. 1.

EFSA, 2007), as in the JECFA evaluation (2004). The AFC Panel established an acceptable daily intake (ADI) of 2 mg/kg bw per day based on the application of a 100-fold safety factor to the NOAEL of 200 mg/kg bw from a 52-week dog study. The AFC Panel did not consider the effects on feed consumption and body weight, observed in other toxicological studies in rodents, as they were attributed to decreased palatability and not to the toxicity of the compound. The AFC Panel concluded that neotame did not raise safety concerns with respect to the proposed uses as a sweetener and flavour enhancer (EFSA, 2007).

2 | DATA AND METHODOLOGIES

The current risk assessment was carried out by the EFSA Panel on Food Additives and Flavourings (FAF Panel) in the context of Regulation (EC) No 257/2010. Structured protocols on hazard identification and characterisation (EFSA, 2020a; EFSA FAF Panel, 2023) and on exposure assessment (EFSA, 2020b; EFSA FAF Panel, 2024) were developed in line with the principles of the EFSA PROMETHEUS project (PROmoting METHods for Evidence Use in Scientific assessments) (EFSA, 2015). The protocols define the strategy to be applied for collecting and selecting data, appraising the relevant evidence, and analysing and integrating the evidence in order to draw conclusions that will form the basis for the scientific opinions.

The draft protocol for the hazard identification and characterisation of sweeteners was published on EFSA's website for comments, and the online public consultation was made available until 19 September 2019. A technical report on the outcome of this public consultation with the overview of the comments received and the general responses from EFSA was published (EFSA, 2020a). During the implementation phase, some amendments and further elaborations to the original protocol were introduced. The changes introduced are documented in the revised version published in 2023 (EFSA FAF Panel, 2023) and were followed for the preparation of the present opinion.

The draft protocol for assessing dietary exposure to sweeteners was published on EFSA's website for comments, and the online public consultation was made available until 22 November 2019. A technical report on the outcome of this public consultation with the overview of the comments received and the general responses from EFSA was published (EFSA, 2020b). The protocol was revised and the changes introduced are documented in the revised version published in December 2024 (EFSA FAF Panel, 2024).

2.1 | Data

The FAF Panel was not provided with a newly submitted dossier for the re-evaluation of neotame (E 961). In accordance with Regulation (EU) No 257/2010, EFSA launched public calls for data[4,5,6,7] and contacted interested parties that had replied to the call for data to collect additional clarification or supplemental information (Documentation provided to EFSA n. 1–10).

The Panel based its assessment on information submitted to EFSA following the public calls for data, information from previous evaluations and additional available literature, up to 2 October 2024. The steps followed for the data acquisition and their selection are documented in Appendix B.

Food consumption data used to estimate the dietary exposure to neotame (E 961) were derived from the EFSA Comprehensive European Food Consumption Database[8] (Comprehensive Database). The Mintel's Global New Products Database (GNPD) was checked to identify the uses of neotame (E 961) in food and beverage products and food supplements. The Mintel's GNPD is an online database that contains the compulsory ingredient information present on the label of numerous products.

2.2 | Methodologies

This opinion was formulated following the principles described in the EFSA Guidance on transparency with regard to scientific aspects of risk assessment (EFSA Scientific Committee, 2009) and following the relevant existing guidance documents from the EFSA Scientific Committee. In line with these principles, this risk assessment was carried out based on structured protocols on hazard identification and characterisation of sweeteners (EFSA, 2020a; EFSA FAF Panel, 2023) and on exposure assessment (EFSA, 2020b; EFSA FAF Panel, 2024).

The FAF Panel assessed the safety of neotame (E 961) as a food additive in line with the principles laid down in Regulation (EU) 257/2010 and in the relevant guidance documents: Guidance on submission for food additive evaluations by the

[4]Call for technical and toxicological data on sweeteners authorised as food additives in the EU - Extended deadline for submitting data: 30/6/2018: https://www.efsa.europa.eu/en/data/call/170621.

[5]Call for technical data on sweeteners authorised as food additives in the EU: https://www.efsa.europa.eu/en/consultations/call/call-technical-data-sweeteners-authorised-food-additives-eu.

[6]Call for food additives usage level and/or concentration data in food and beverages intended for human consumption (Batch 7).

[7]Call for data on genotoxicity data on sweeteners: https://www.efsa.europa.eu/en/call/call-data-genotoxicity-data-sweeteners.

[8]https://www.efsa.europa.eu/en/data-report/food-consumption-data.

Scientific Committee on Food (SCF, 2001) and the Guidance for submission for food additive evaluations in 2012 (EFSA ANS Panel, 2012).

In animal studies, when the test substance is administered in the feed or in the drinking water, but doses are not explicitly reported by the authors as mg/kg bw per day based on actual feed or water consumption, the daily intake is calculated by the Panel using the relevant default values. In case of rodents, the values as indicated in the EFSA Scientific Committee Guidance document (EFSA Scientific Committee, 2012) are applied. In the case of other animal species, the default values used by JECFA (2000) are used. In these cases, the dose is expressed as 'equivalent to mg/kg bw per day'. If a concentration in feed or drinking water was reported and the dose in mg/kg bw per day was calculated (by the authors of the study report or the Panel) based on these reported concentrations and on reported consumption data for feed or drinking water, the dose is expressed as 'equal to mg/kg bw per day'. When in adult human studies (aged above 18 years) the dose of the test substance administered was reported in mg/person per day, the dose in mg/kg bw per day is calculated by the Panel using a body weight of 70 kg as default for the adult population as described in the EFSA Scientific Committee Guidance document (EFSA Scientific Committee, 2012).

No new data were received following the call for biological and toxicological data.[6] From the literature search performed,[9] the new studies which were considered as relevant, according to the inclusion criteria reported in the protocol on hazard identification and characterisation (EFSA, 2020a; EFSA FAF Panel, 2023), are either reported in Sections 3.5.6, 3.5.7 or Appendix B (Table B). In the safety evaluation of neotame (E 961) by EFSA (2007) as a basis for its authorisation, the AFC Panel had reviewed several toxicological studies in animal and human studies available at that time. In this opinion, a summary of these studies is presented and more details can be found in the 2007 EFSA opinion. In accordance with the revised protocol (EFSA FAF Panel, 2023), studies previously evaluated by the SCF or EFSA and considered for setting an ADI were also subjected to a RoB evaluation. In the case of neotame, the study on which the ADI was based (i.e. the 52-week dog study, cited in EFSA, 2007 as PCR 1017) was considered of moderate risk of bias (RoB) (allocated as Tier 2, see Appendix B). Therefore, according to the criteria outlined in the revised protocol the Panel considered it appropriate not to re-assess the other previously considered studies. The methods followed to perform this risk assessment are detailed in Appendix B.

Dietary exposure to neotame (E 961) from its use as a food additive was estimated by combining food consumption data available within the EFSA Comprehensive Database with the maximum levels according to Annex II to Regulation (EC) No 1333/2008[10] and with reported use levels and analytic levels submitted to EFSA following a call for data. The exposure was calculated according to different scenarios (see Section 3.4).

Finally, uncertainties in the hazard identification, characterisation and exposure assessment were identified and discussed exposure assessment were identified and discussed (see Section 4).

3 | ASSESSMENT

3.1 | Technical data

3.1.1 | Identity of the substance and specifications

Neotame is the chemically manufactured compound N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester and is authorised as a sweetener (E 961). The chemical structure of neotame (E 961) is given in Figure 1.

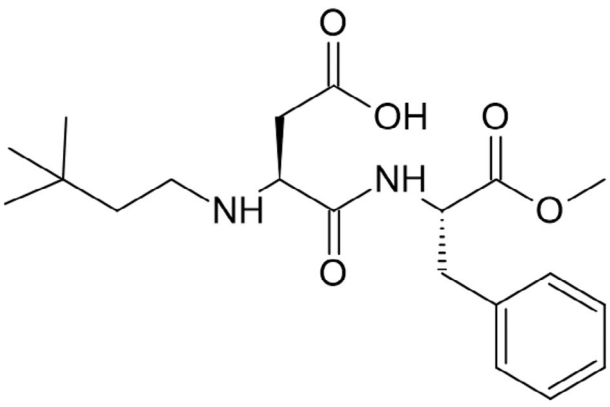


FIGURE 1 Chemical structure of neotame (E 961).

[9]To cover the period from the latest EFSA evaluation with an overlap of 1 year (i.e. 2006) until 2 October 2024.

[10]Commission 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. 16.

Specifications for neotame (E 961) as laid down in the Commission Regulation (EU) No 231/2012 and by JECFA (2003) are listed in Table 1, including information on the manufacturing process of neotame (E 961).

TABLE 1 Specifications for neotame (E 961) according to Commission Regulation (EU) No 231/2012 and proposed by JECFA (2003).

	Commission regulation (EU) No 231/2012	JECFA (2003)
Synonyms	N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester; N(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methyl ester	INS No. 961
Definition	Neotame is manufactured by reaction under hydrogen pressure of aspartame with 3,3-dimethylbutyraldehyde in methanol in presence of a palladium/carbon catalyst. It is isolated and purified by filtration, where diatomaceous earth may be used. After solvent removal via distillation, neotame is washed with water, isolated by centrifugation and finally vacuum dried.	Neotame is manufactured in single process in which aspartame and 3,3-dimethylbutyraldehyde are reacted together in a methanol solution in the presence of hydrogen. Neotame is isolated by removal of methanol, followed by washing and drying.
CAS No.	165450-17-9	165450-17-9
Chemical name	N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester	N-[N-(3,3-Dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester
Chemical formula	$C_{20}H_{30}N_2O_5$	$C_{20}H_{30}N_2O_5$
Molecular weight	378.47	378.47
Description	White to off-white powder	White to off-white powder
Assay	Not less than 97.0% on the dried basis	Not less than 97.0% and not more than 102.0% on the anhydrous basis.
Identification		
Solubility	4.75% (w/w) at 60°C in water, soluble in ethanol and ethyl acetate	Sparingly soluble in water, very soluble in ethanol.
Infrared absorption spectrum:	–	The infrared spectrum of a potassium bromide dispersion of the sample corresponds to the standard infrared spectrum in Appendix A
Purity		
Water content	Not more than 5% (Karl Fischer, sample size 25 ± 5mg)	Not more than 5.0% in a sample size of 25 ± 5 mg (Karl Fischer)
pH	5.0–7.0 (0.5% aqueous solution)	5.0–7.0 (0.5% solution)
Melting range	81–84°C	81–84°
N-[(3,3-dimethylbutyl)-L-α-aspartyl]-L- phenylalanine	Not more than 1.5%	Not more than 1.5%
Other related substances	–	Not more than 2.0%
Lead	Not more than 1 mg/kg	Not more than 1 mg/kg
Sulphated ash	–	Not more than 0.2%
Specific rotation	–	$[\alpha]_D^{20}$ Between −40.0° and −43.3° (0.5% solution) calculated on the anhydrous basis

Following the EFSA calls for technical data[11,12] one interested business operator (IBO) provided data and information to support the re-evaluation of neotame (E 961) and the compliance with the current EU specifications, as laid down in Commission Regulation (EU) No 231/2012. Technical data on commercial samples of neotame (E 961) supported by certificates of analysis, were provided by this IBO (Documentation provided to EFSA n. 1, 2, 3, 5, 7, 8).

Organic impurities

The main impurity of neotame (E 961) is also a degradation product (de-esterified form) N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751) (Table 2). A limit of 'Not more than 1.5 %' is set for this impurity in the EU specifications. The Panel noted that in the EU specifications, this impurity is referred as N-[(3,3-dimethylbutyl)-L-α-aspartyl]-L- phenylalanine

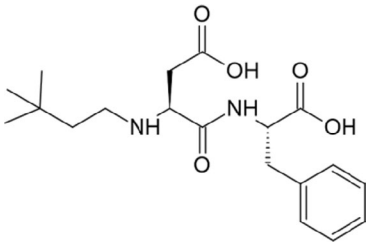
[11]Call for technical and toxicological data on sweeteners authorised as food additives in the EU – Extended deadline for submitting data: 30/6/2018. Published: 21 June 2017. Available from: https://www.efsa.europa.eu/en/data/call/170621.

[12]Call for technical data on sweeteners authorised as food additives in the EU. Published: 13 May 2019. Available from: https://www.efsa.europa.eu/en/consultations/call/call-technical-data-sweeteners-authorised-food-additives-eu.

and the name should be changed to N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine, to be consistent with the chemical name of the food additive itself.

The Panel noted that apart from NC-00751, the parameter 'other related substances' (Not more than 2.0%) as described in the specifications of JECFA (2003), is not included in the current EU specifications (Table 1). Such structurally related impurities of E 961 are included in Appendix A of the EFSA AFC Panel (2007) and in Appendix D, Table D.1 of the current opinion.

TABLE 2 Main impurity of neotame (E 961).

Chemical name/IUPAC name	CAS No.	Structural formula
N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (3S)-4-[[(1S)-1-carboxy-2-phenylethyl]amino]-3-(3,3-dimethylbutylamino)-4-oxobutanoic acid NC-00751	190910-14-6	

Ten samples of the food additive were analysed using high-performance liquid chromatography with an ultraviolet detector (HPLC-UV). Neotame, its degradation product N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine, and 'other related substances' were reported at levels of 98.2%–100.9%, 0.10%–0.42% and 0.05%–0.19%, respectively (Documentation provided to EFSA n. 1, 3).

Considering the manufacturing process provided by the IBO (see Section 3.1.4), the food additive is a product of chemical synthesis using well defined chemical precursors, involving several separation and purification steps. The analytical data provided for the purity of neotame (E 961) were in all analysed samples higher than 98%. This being the case, the Panel recommends raising the minimum percentage of neotame from 97% to at least 98% in the assay requirement outlined in the EU specifications.

The Panel noted that the specified impurity N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine is the major degradation product (see also section Stability) and the major metabolite of neotame (see section ADME). Therefore, the safety of this impurity is covered by the toxicity data available on neotame (see section ADME and Genotoxicity) and does not give rise to concern as an impurity at the level of up to 1.5%.

The potential exposure to the 'other related substances', resulting from the use of E 961 was individually assessed using the threshold of toxicological concern (TTC) approach (Appendix E). The 'other related substances' (Appendix D, with the exception of L-phenylalanine and 3,3-dimethylbutylamine (Cramer Class I)) were classified into Cramer Class III (Appendix F, Table F.2). In order not to exceed the respective TTC value (1.5 μg/kg bw per day), their presence individually should be less than ~0.9% in the food additive. Considering the exposure estimates, the purity value of neotame and the analytical data provided for the 'other related substances', the Panel noted that no concern is identified for those impurities and thus, there is no need for them to be included in the EU specifications.

Other purity parameters

The IBO provided analytical data on 10 samples of the food additive on water content (2.70%–4.50%, Karl-Fisher titrimetric method), residue on ignition (sulphated ash) (0.01%–0.06%, gravimetric method, 550°C) and specific optical rotation (−41.4 to −42.6° at 20°C, 0.5% solution on dry basis). All analyses were performed according to the U.S. Pharmacopeia, Food Chemicals Codex 7th Edition, 2010–2011 (Documentation provided to EFSA n. 1, 3).

Since neotame (E 961) is optically active, the Panel considered that information on its specific optical rotation should be included in the EU specifications.

Toxic elements

Regarding toxic elements, the IBO provided analytical data on the levels of lead (Pb), mercury (Hg), cadmium (Cd), arsenic (As) and palladium (Pd) in different analysed samples (Documentation provided to EFSA n. 1, 3, 7, 8). Details of the analytical data provided are available in Appendix A. The Panel noted that no information on the lowest technologically achievable levels for the toxic elements in E 961 was provided by the IBO, as requested in the relevant call for data.

The Panel noted that among the potential inorganic impurities tested, only lead has a defined limit value in EU specifications. Taking into account the data provided additionally for arsenic, cadmium and mercury and taking also into account that the manufacturing process is an organic synthesis with several separation and purification steps and so systematic contamination by these inorganics is not anticipated, the Panel did not see a need to recommend additional specifications for arsenic, cadmium and mercury.

The Panel assessed the risk that would result if (i) Pb was present in E 961 at the current maximum limit in the EU specifications and if (ii) Pd was present in E 961 at the rounded up highest measured value provided by one IBO. The outcome of the risk assessment for these scenarios is presented in Table A.1, Appendix A.

The Panel noted that the choice of maximum limits for toxic elements in the EU specifications is in the remit of risk management.

Microbiological parameters

The Panel noted that according to Commission Regulation (EU) No 231/2012, no microbiological specifications are currently set for E 961. Noting the nature of the food additive (E 961) and the various steps of the manufacturing process (Section 3.1.4 Manufacturing process), the Panel considered that a microbiological contamination is unlikely. This was supported by the microbiological data (aerobic plate count, fungi, total coliforms and *E. coli*) in 5 samples of the food additive submitted by the interested business operator (IBO) (Documentation provided to EFSA n. 3). Hence, the Panel did not consider it necessary to recommend inclusion of microbiological criteria in the EU specifications for E 961.

3.1.2 | Solubility

One IBO provided data on the solubility of neotame (E 961) in water, ethanol and ethyl acetate at different temperatures (15, 25, 40, 50 and 60°C). For the determination of solubility, saturated solutions of neotame were prepared in triplicate in 100 g of each solvent. The solutions were stirred in a temperature controlling bath, until the solid was visually dissolved. The solutions were then left undisturbed for 60 ± 10 mins in order for the undissolved neotame to precipitate. The concentration of neotame in the saturated solutions was quantified using an high-performance liquid chromatography with ultraviolet detection (HPLC–UV) method. The solubility results are provided in Table 3. (Documentation provided to EFSA n. 3, 5).

The Panel noted that the ultrafiltration step recommended in the EFSA Guidance on Particle-TR (EFSA Scientific Committee, 2021b), to remove any small particles potentially present in the solubilised fraction, was not included in these tests for solubility.

TABLE 3 Results of the solubility tests of neotame (E 961) in different solvents (Documentation provided to EFSA n. 3).

	Mean (*n* = 3) solubility (g/100g of solvent)		
Temperature (°C)	**Water**	**Ethyl acetate**	**Ethanol**
15	1.06	4.36	> 100
25	1.26	7.70	> 100
40	1.80	23.8	> 100
50	2.52	87.2	> 100
60	4.75	> 100	> 100

The Panel noted that the current EU specifications include a solubility value of *'4.75% (w/w) at 60°C in water, and solubility in ethanol and ethyl acetate'*, while in the JECFA specifications a description related to the solubility only for water and ethanol is included as '*sparingly soluble in water, very soluble in ethanol*'. According to the analytical data provided by the IBO, neotame (E 961) can be considered sparingly soluble in water in the range of the temperatures 15–25°C and very soluble in ethanol. Therefore, the Panel suggests an amendment to the description of the solubility of E 961 in line with the JECFA specifications.

In addition, the Panel took note of the observation made by one IBO that *'ethyl acetate is not a particularly useful solvent for food applications, nor for analytical purposes'* (Documentation provided to EFSA n. 5) and concurred with the suggestion by that IBO to remove the provision on solubility in ethyl acetate from the EU specifications of E 961.

3.1.3 | Particle size

One IBO provided a laser diffraction (LD) analysis for three samples of E 961 (Documentation provided to EFSA n. 2). The method was not described; density distribution curves were reported. The Panel noted that LD analysis is not considered a proper method to investigate the presence of small particles including nanoparticles as it does not provide information on the size of the constituent particles as required by the Guidance on Particle-TR and it is prone to errors for polydisperse materials.

A transmission electron microscopy (TEM) analysis of one sample of neotame (E 961) was provided by one IBO (Documentation provided to EFSA n. 3). The method was not well described, and quantitative statistics were not provided. Based on a series of transmission electron micrographs the IBO reported that '*the particles were generally in a range from*

around a micrometer up to tens of micrometer. The particles did show evidence of smaller features within them, however they appeared to be fused to and an integral part of the particles themselves'.

The Panel identified several drawbacks of the performed TEM analysis such as the sample preparation by sprinkling the dry powdered material '*on a holey carbon film*'; this type of specimen preparation resulted in the deposition of relatively large particles onto the EM-grid. The thickness of these particles does not allow to demonstrate, using the TEM method, whether these large particles consist of agglomerated or aggregated constituent particles. Furthermore, the resolution of the electron micrographs is not reported, and it does not allow to visualise particles in the nanoscale.

The IBO also provided information on the particle size distribution for three samples of E 961 based on the analysis of the sub-micrometre < 1 μm particles fraction by Nanoparticle Tracking Analysis (NTA) (Documentation provided to EFSA n. 2). The method was not well described (for example, information on the carrier medium and the dispersion protocol was missing), and the particle size distribution in number and quantitative statistics were not provided. For the three tested samples the IBO reported the d10 ranging from 36 to 57.4 nm, d50 ranging from 71.4 to 103.5 nm and d90 ranging from 119 to 287.6 nm. The analysis was performed at the concentration 15 g/L[13] which is above the water solubility of the food additive (12.6 g/L at 25°C).

Based on the submitted data, the Panel noted that the presence of small particles including nanoparticles in the food additive E 961 has been reported by the IBO under the examined conditions.

3.1.4 | Manufacturing process

Following the EFSA call for technical data (2017), no new information on the manufacturing process for neotame (E 961) has been submitted, apart from what was previously described in the relevant EFSA opinion (EFSA, 2007).

According to the previous EFSA opinion on neotame (E 961) (EFSA, 2007), neotame is manufactured from N-L-α-aspartyl-L-phenylalanine 1-methyl ester (aspartame) and 3,3-dimethylbutyraldehyde via reductive amination. The starting substances are dissolved in methanol, under hydrogen pressure, at room temperature for several hours. The reaction is catalysed by palladium on carbon. The catalyst is then removed by filtration. Diatomaceous earth can be used to facilitate the filtration phase. The isolation and purification of neotame is performed by distilling off the methanol followed by addition of water. The resulting mixture is cooled down for a number of hours. Then, neotame is isolated by centrifugation, washed with water and vacuum dried. The product may be milled to obtain the desired particle size (Figure 2).

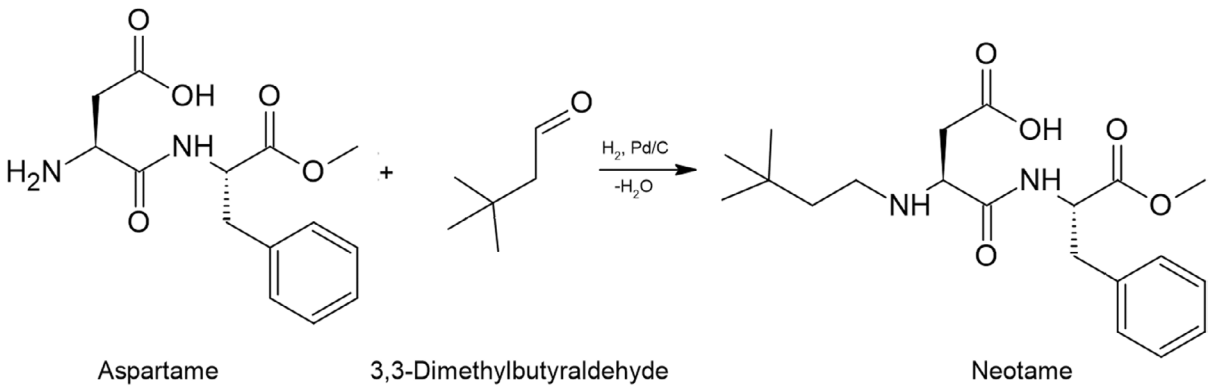


FIGURE 2 Neotame (E 961) synthesis scheme.

The Panel noted after a systematic literature search, that the manufacturing of the neotame precursor N-(3,3-dimethylbutyl)-L-aspartic acid via the use of a biocatalyst (engineered C–N Lyase) is described (Zhang et al., 2020). However, no IBOs have indicated the use of this manufacturing process, therefore, the biocatalytic synthesis of E 961 has not been assessed by the Panel.

3.1.5 | Methods of analysis in food

Following the EFSA call for technical data (2017), no new information on methods of analysis for neotame (E 961) has been submitted, apart from what was submitted at the time of the relevant EFSA assessment in 2007 (EFSA, 2007).

Information on a reversed-phase high-performance liquid chromatography (RP-HPLC) method to analyse neotame in food and beverages was provided by one IBO (Documentation provided to EFSA n. 1). The HPLC method aims to quantify neotame (E 961), and the main degradation product N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751) in

[13]The Panel assumed that the carrier used for the analysis was water.

powdered sweetener formulations (tabletop powder) and in several food and beverage applications using method modifications according to the food matrix. The detection is performed by an ultraviolet (UV) detector at 210 nm.

Several publications on the development of analytical methods for neotame determination, or simultaneous determination of different sweeteners, in food and beverage matrices were also retrieved following a systematic literature search. The most applied technique for analytical determination of neotame is the chromatographic separation with HPLC system coupled with a broad variety of detection systems, in particular diode array detection (DAD) (Kumari, Arora, et al., 2016; Kumari, Choudhary, et al., 2016; Lorenzo et al., 2015; Sezgin et al., 2021), evaporative light scattering detection (ELSD) (de Sousa et al., 2024; Soyseven et al., 2023), charged aerosol detection (CAD) (Cheng et al., 2024), ultraviolet detection (UV) (Zhu et al., 2023), mass spectrometric detection (MS) (Zygler et al., 2010; Lorenzo et al., 2015; Henschel et al., 2016; Kokotou & Thomaidis, 2018; Krmela et al., 2020; Zhu et al., 2023), matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Wang et al., 2024), quadrupole-trap mass spectrometry (QTRAP MS) and tandem MS detection (MS/MS) (Cheng et al., 2024; Detry et al., 2022; Hu et al., 2023; Iwakoshi et al., 2019; Li et al., 2020; Nicoluci et al., 2023; Shah et al., 2014).

Other types of techniques occasionally applied for the determination of neotame are electrochemical determination and capillary electrophoresis (Bathinapatla et al., 2014). The latter permits the separation of the neotame diastereoisomers by the use of cyclodextrins as chiral selector (Hu et al., 2013). Lephalala et al. (2020) described the development of an enzymatic biosensor to detect and quantify neotame in soft drinks. Han et al. (2020) described the identification and quantification of neotame in instant grain beverages, based on surface-enhanced Raman scattering (SERS) technique and filter paper-based silver nanoparticles (AgNPs@FP) substrates.

3.1.6 | Stability of the substance and reaction and fate in food

Following the EFSA call for technical data (2017), no new information or studies to investigate the stability of neotame (E 961) has been submitted, apart from what was previously described in the EFSA opinion (EFSA AFC Panel, 2007).

Extensive information regarding the stability and the formation of possible reaction products is described in the technical part (Appendix A: Identification of key compounds) of the previous EFSA scientific opinion on the evaluation of neotame as a sweetener and flavour enhancer (EFSA AFC Panel, 2007). These data are summarised below.

The stability of neotame in bulk dry form, stored at 25°C and 60% relative humidity (RH) for up to 78 weeks was examined, and neotame was quantified within the range of 99.7% to 100.9%, while N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine (NC-00751) levels were ≤ 0.3%. The stability of three additional lots of neotame was measured after 52 weeks of storage at 40°C and 75% RH. The results showed that the neotame content decreased to between 96.2% and 99.0%, while NC-00751 increased from ≤ 0.4% at week 14 to 1.2%–3.5% at week 52. The only additional degradation product detected was N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine (NC-00769) (**Figure 3**) at levels of ≤ 0.2% during the storage period.

To predict the fate of neotame in food products, aqueous solutions of neotame under different normal and extreme conditions were prepared and stored for 26 weeks. Model beverages were formulated with 200 mg/L of neotame in an aqueous solution at pH 3.2 (used as a model for carbonated soft drinks, but neotame was added in high concentration). The concentration of neotame was set at 200 mg/L to ensure the determination of the 13 degradation products reported in Appendix D, as they would not be detected at the actual use levels (15–17 mg/L). 89% of the initial level of neotame remained unchanged after 8 weeks of storage at 20°C. The results showed that neotame slowly degrades mainly through hydrolysis of the methyl ester group which results in equimolar amounts of the de-esterified form N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751) (7.4%) and methanol. Three other minor degradation products (each representing < 1% of the initial concentration of neotame) were formed: N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine 1-methyl ester (NC-00777) formed by cyclisation of neotame (0.82%), N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine 1-methyl ester (NC00764) formed by β-rearrangement of NC-00777 (0.97%) and N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine (NC-00779) formed by methyl ester hydrolysis of NC-00777 (below the LOQ of 1%) (**Figure 3**). The nine remaining impurities were reported as below 0.5% each. In the experimental studies performed in model beverages at the use levels in commercial beverages (15 mg/L) these three minor degradation compounds were not detected. The use of higher concentrations in the stability studies is justified on the basis of similar kinetic behaviour of neotame at 200 mg/L and at 15 mg/L. More information on the study is available in the AFC Panel output of 2007.

An example of the degradation pathway of neotame is given in **Figure 3**.

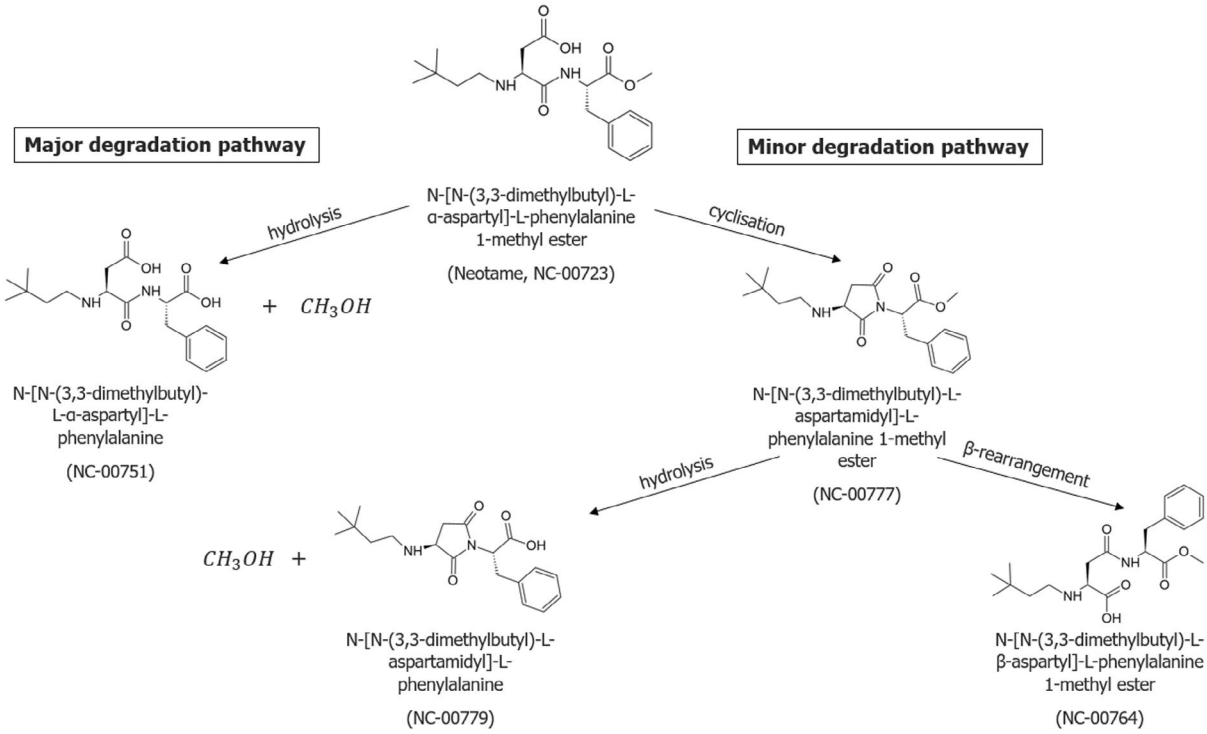


FIGURE 3 Neotame degradation pathway.

Data on stability and fate in food, including the potential neotame degradation products, are also available in the 61st JECFA Chemical and Technical Assessment (CTA) report (JECFA, 2004). According to the CTA of JECFA, neotame being a secondary amine would be able to react with reducing sugars in the food matrix and thus Maillard reactions could occur. However, considering that neotame has a hydrophobic side and that at a pH of 6 or less, which is typical of most foods, the amount of unprotonated amine is less than 0.02%, the extent of the Maillard reaction would be expected to be small. Therefore, JECFA concluded that Maillard reaction with neotame would not lead to a significant amount of degradation products. The FAF Panel agreed with this conclusion.

The experimental investigations on the chemical stability of neotame, under conditions of intended use as a sweetener, showed that the stability of the food additive was pH, temperature and time dependent. The rate and extent of degradation was higher at extreme pH values and higher temperatures with degradation occurring after long storage times. Neotame is relatively stable at pH levels from 3.0 to 5.5 with an optimum stability at pH 4.5 (Nofre & Tinti, 2000).

Papers dealing with neotame stability in food were retrieved from the systematic literature search. Stability studies on neotame have been performed in yoghurt, during its processing, fermentation and storage (Kumari et al., 2017), in cake and ice cream (Kumari et al., 2023; Kumari, Arora, et al., 2016) and in pasteurised and in-bottle sterilised flavoured milk (Kumari, Choudhary, et al., 2016). Results showed that neotame was stable in milk under heating conditions (85°C/30 min) with losses of 2.3%, and no significant loss at high temperature short time conditions (88°C/30 s). In heat-treated flavoured milk (90°C/20 min) losses of 8% were reported while at 68°C/30 min resulted in no degradation of neotame in an ice cream mix. However, the neotame content decreased from 99.4% to 89.9%, during storage (−18°C/90 days) of ice cream. At higher temperatures (> 100°C), more neotame degradation was observed during flavoured milk heating (121°C/15 min) than at cake-baking temperature (180°C/20 min), with 50% and 13% loss, respectively. During yoghurt storage (4–7°C/15 days) there was no significant loss of neotame. Prakash et al. (2002) also reported that about 98% of the initial neotame remained in yoghurt at the end of 6-week periods. During storage, heat-treated flavoured milk (60 days at 30°C), presented an appreciable loss of neotame (from 50.36% to 8.67%). During storage of cake a loss of 29% of neotame was observed after 20 days of storage. Stability of neotame was not affected by the fermentation conditions in yoghurt (42°C for about 4 h until pH 4.5 and acidity 0.75 g/100 mL lactic acid).

The Panel noted that, based on the submitted information along with considerations of the structure and characteristics of neotame, E 961 is stable in most food categories, but is prone to hydrolysis and other degradation pathways, particularly at very low pH and high temperature, to form mainly NC-00751, along with NC-00777, NC-00779 and NC00764, plus other minor degradation products (Appendix D).

3.2 | Authorised uses and use levels

Maximum levels of neotame (E 961) are defined in Annex II to Regulation (EC) No 1333/2008[14] on food additives, as amended. In this document, these levels are called maximum permitted levels (MPLs).

Currently, neotame (E 961) is an authorised food additive in the EU in 34 food categories: 31 food categories with MPLs ranging from 1 to 250 mg/kg and three food categories at quantum satis (QS) in tabletop sweeteners. Table 4 lists the food categories with their restrictions/exceptions that are permitted to contain added neotame (E 961) and the corresponding MPLs as set by Annex II to Regulation (EC) No 1333/2008.

TABLE 4 MPLs of neotame in foods according to Annex II to Regulation (EC) No 1333/2008.

Food category number	Food category name	Restrictions/exception	MPL (mg/L or mg/kg as appropriate)
01.4	Flavoured fermented milk products including heat-treated products	Only energy-reduced products or with no added sugar	32
03	Edible ices	Only energy-reduced or with no added sugar	26
04.2.2	Fruit and vegetables in vinegar, oil or brine	Only sweet–sour preserves of fruit and vegetables	10
04.2.3	Canned or bottled fruit and vegetables	Only fruit energy-reduced or with no added sugar	32
04.2.4.1	Fruit and vegetable preparations excluding compote	Only energy-reduced	32
04.2.5.1	Extra jam and extra jelly as defined by Directive 2001/113/EC	Only energy-reduced jams, jellies and marmalades	32
04.2.5.1	Extra jam and extra jelly as defined by Directive 2001/113/EC	Only energy-reduced jams, jellies and marmalades, as flavour enhancer	2
04.2.5.2	Jam, jellies and marmalades and sweetened chestnut puree as defined by Directive 2001/113/EC	Only energy-reduced jams, jellies and *marmalades*	32
04.2.5.2	Jam, jellies and marmalades and sweetened chestnut puree as defined by Directive 2001/113/EC	Only energy-reduced jams, jellies and marmalades, as flavour enhancer	2
04.2.5.3	Other similar fruit or vegetable spreads	Only energy-reduced fruit or vegetable spreads and dried fruit-based sandwich spreads, energy-reduced or with no added sugar	32
05.1	Cocoa and Chocolate products as covered by Directive 2000/36/EC	Only energy-reduced or with no added sugar	65
05.2	Other confectionery including breath refreshening microsweets	Only cocoa or dried fruit based, energy reduced or with no added sugar	65
05.2	Other confectionery including breath refreshening microsweets	Only energy-reduces tablet form confectionary	15
05.2	Other confectionery including breath refreshening microsweets	Only cocoa, milk, dried fruit or fat based sandwich spreads, energy-reduced or with no added sugar	32
05.2	Other confectionery including breath refreshening microsweets	Only starch-based confectionery energy reduced or with no added sugar	65
05.2	Other confectionery including breath refreshening microsweets	Only starch-based confectionery energy reduced or with no added sugar, as flavour enhancer	3
05.2	Other confectionery including breath refreshening microsweets	Only confectionery with no added sugar	32
05.2	Other confectionery including breath refreshening microsweets	Only breath-freshening microsweets, with no added sugar	200
05.2	Other confectionery including breath refreshening microsweets	Only breath-freshening microsweets and strongly flavoured throat pastilles with no added sugar, as flavoured enhancer	3
05.2	Other confectionery including breath refreshening microsweets	Only strongly flavoured throat pastilles with no added sugar	65
05.3	Chewing gum	Only with added sugars or polyols, as flavour enhancer	3
05.3	Chewing gum	Only with no added sugar	250

[14]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. 16.

TABLE 4 (Continued)

Food category number	Food category name	Restrictions/exception	MPL (mg/L or mg/kg as appropriate)
05.4	Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4	Only starch-based confectionery energy reduced or with no added sugar	65
05.4	Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4	Only starch-based confectionery energy reduced or with no added sugar, as flavour enhancer	3
05.4	Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4	Only confectionery with no added sugar	32
05.4	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	65
05.4	Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4	Only sauces	12
05.4	Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4	Only sauces as flavour enhancer	2
06.3	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	32
07.2	Fine bakery wares	Only cornets and wafers, for ice cream, with no added sugar	60
07.2	Fine bakery wares	Only *essoblaten* – wafer paper	60
09.2	Processed fish and fishery products including molluscs and crustaceans	Only sweet–sour preserves and semi-preserves of fish and marinades of fish, crustaceans and molluscs	10
11.4.1	Tabletop Sweeteners in liquid form		QS
11.4.2	Tabletop Sweeteners in powder form		QS
11.4.3	Tabletop Sweeteners in tablets		QS
12.4	Mustard		12
12.5	Soups and broths	Only energy-reduced soups	5
12.6	Sauces		12
12.6	Sauces	Only as flavour enhancer	2
12.7	Salads and savoury based sandwich spreads	Only *Feinkostsalat*	12
13.2	Dietary foods for special medical purposes defined in Directive 1999/21/EC (excluding products from food category 13.1.5)		32
13.3	Dietary foods for weight control diets intended to replace total daily food intake or an individual meal (the whole or part of the total daily diet)		26
14.1.3	Fruit nectars as defined by Directive 2001/112/EC and vegetable nectars and similar products	Only energy-reduced or with no added sugar	20
14.1.4	Flavoured drinks	Only energy reduced or with no added sugar	20
14.1.4	Flavoured drinks	Only energy reduced or with no added sugar, as flavour enhancer	2
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	20
14.2.1	Beer and malt beverages	Only energy-reduced beer	1
14.2.3	Cider and perry		20
14.2.8	Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15% of alcohol		20
15.1	Potato-, cereal-, flour- or starch-based snacks		18

(Continues)

TABLE 4 (Continued)

Food category number	Food category name	Restrictions/exception	MPL (mg/L or mg/kg as appropriate)
15.1	Potato-, cereal-, flour- or starch-based snacks	As flavour enhancer only	2
15.2	Processed nuts		18
15.2	Processed nuts	As flavour enhancer only	2
16	Desserts excluding products covered in category 1, 3 and 4	Only energy-reduced or with no added sugar	32
17.1	Food supplements supplied in a solid form, excluding food supplements for infants and young children		60
17.1	Food supplements supplied in a solid form, excluding food supplements for infants and young children	Only food supplements in chewable form	185
17.1	Food supplements supplied in a solid form, excluding food supplements for infants and young children	Only as flavour enhancer, except food supplements in chewable form	2
17.1	Food supplements supplied in a solid form, excluding food supplements for infants and young children	Only vitamins and/or mineral based food supplements in chewable form, as a flavour enhancer	2
17.2	Food supplements supplied in a liquid form, excluding food supplements for infants and young children		20
17.2	Food supplements supplied in a liquid form, excluding food supplements for infants and young children	Only food supplements in syrup form	185
17.2	Food supplements supplied in a liquid form, excluding food supplements for infants and young children	Only as flavour enhancer, except food supplements in syrup form	2
17.2	Food supplements supplied in a liquid form, excluding food supplements for infants and young children	Only vitamins and/or mineral based food supplements in syrup form, as a flavour enhancer	2

Abbreviations: MPL, maximum permitted level; QS, *Quantum Satis*.

Neotame (E 961) is not authorised according to Annex III of Regulation (EC) No 1333/2008.

3.3 | Exposure data

3.3.1 | Concentration data

Most food additives in the EU are authorised at a specific MPL. However, a food additive may be used at a lower level than the MPL. Therefore, actual concentration data are required to perform a more realistic exposure assessment as well as to obtain data for those food categories authorised at QS.

In the framework of Regulation (EC) No 1333/2008 on food additives and of Commission Regulation (EU) No 257/2010 regarding the re-evaluation of approved food additives, and specifically sweeteners, EFSA issued one public call[11] for concentration data (use levels and/or analytical data) in sweeteners, including neotame (E 961). Usage level data on neotame (E 961) in food and beverages submitted by two industry stakeholders through the call[11] and analytical data by four Member States as available in EFSA's DWH on the 3 October 2024, were considered for the present assessment.

Reported use levels of neotame (E 961)

Industry provided two use levels for neotame (E 961), one in sugar free candies belonging to the food category (FC) 05.2 Other confectionery including breath-freshening microsweets, and one in sugar free fruit nectars belonging to FC 14.1.3 Fruit nectars as defined by Directive 2001/112/EC and vegetable nectars and similar products. No specific use levels related to the restrictions/exemptions in the authorised uses of these two food categories (Table 4) were provided. The use level reported for fruit nectars referred to a niche product. In the refined exposure scenario, niche product data are considered if there are no other concentration data (use level or analytical data) available for more widely consumed products belonging to the same food category. In this case, the use level in the niche product fruit nectars was the only available use level for this category. Therefore, it was retained in the refined scenario.

The reported use levels were provided by the Association of Italian Sweets and Pasta Industries and the European Fruit Juice Association and are listed in Table A.1 of Annex A.

Summarised data on analytical results of neotame (E 961) provided by Member States

Twelve analytical results of neotame (E 961) for FC 5.3 Chewing gums without added sugar were submitted by Belgium with concentrations ranging from 14 to 50 mg/kg and an average of 27.9 mg/kg. One result for FC 14.1.4 Flavoured drinks was provided by Slovakia at a concentration of 2.8 mg/kg. Three analytical results for FC 5.2 Other confectionery including breath-freshening microsweets were submitted by Belgium ($n=2$) and Italy ($n=1$) with concentrations ranging from 28 to 50 mg/kg and an average of 37.7 mg/kg. One analytical result for FC 17.2 Food supplements supplied in a liquid form, excluding food supplements for infants and young children was submitted by Germany at 7.5 mg/kg and referred to as 'Protein and amino acids supplements'.

Only eating events referring to Protein and amino acids supplements were thus included in the exposure assessment for this category. The concentration in the solid form for Protein and amino acids supplements corresponding to FC 17.1 Food supplements supplied in a solid form, excluding food supplements for infants and young children, was set at the MPL value of 60 mg/kg because applying a conversion factor of 10 to the analytical result for the liquid form would have resulted in a higher concentration (75 mg/kg).

For food categories for which only one analytical result was available the mean and maximum value used in assessment is the same.

Details on the analytical results are provided in Table A.2 of Annex A.

3.3.2 | Summarised data extracted from the Mintel's global new products database

For the purpose of this Scientific Opinion, Mintel's GNPD was used for checking the labelling of food and beverages products and food supplements for neotame (E 961)[15] within the EU's food market as the database contains the compulsory ingredient information on the label. Mintel's GNPD is an online database which monitors new introductions of packaged goods in the market worldwide currently having 24 out of its 27 member countries, and Norway included.

According to Mintel's GNPD, neotame (E 961) was labelled on 57 products between October 2019 and October 2024. These products represented 0.07% of all the products within the food subcategories of Mintel's GNPD food classification in which at least one food was labelled with neotame (E 961).

The food subcategories with the highest number of products labelled with neotame (E 961), were 'Standard & Power Mints' (corresponding to the FC 05.2 Other confectionery including breath refreshening microsweets) and 'Prepared Meals' (corresponding to the FC 18 Processed foods not covered by categories 1 to 17, excluding foods for infants and young children) with 10 and 18 products, respectively. In the subcategory 'Prepared meals' only sushi products were labelled to contain neotame (E 961). Despite this, no use levels or analytical data were submitted to EFSA for food categories that are related to sushi products (FC 9.2 Processed fish and fishery products including molluscs and crustaceans in the case of fish sushi, or FC 4.2.4.1 Fruit and vegetable preparation excluding compote for vegetarian sushi).

Eight products labelled with neotame (E 961) referred to FC 14.1.4 Flavoured drinks and two referred to FC 11.4 Tabletop sweeteners. Details on the data extracted from Mintel's GNPD are listed in Table A.3 of Annex A.

Annex A, Table A.3 also includes a match of the food subcategories to the corresponding food categories in Annex II to Regulation (EC) No 1333/2008, however this linkage is indicative since the food subcategories in Mintel's GNPD only partly correspond to the categories in the relevant legislation

3.3.3 | Food consumption data used for exposure assessment

EFSA Comprehensive European Food Consumption Database

Since 2010, the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) has been populated with national data on food consumption at a detailed level. Competent authorities in the European countries provide 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 on the 'Use of the EFSA) Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011). The version of the Comprehensive database taken into account in this assessment was published in July 2021.[16] Data from EU member states were considered for the estimations.

The food consumption data gathered by EFSA were collected by different methodologies and thus direct country-to-country comparisons should be interpreted with caution. Depending on the food category and the level of detail used for exposure calculations, uncertainties could be introduced owing to possible subjects' underreporting and/or misreporting of the consumption amounts. Nevertheless, the EFSA Comprehensive Database includes the currently best available food consumption data across Europe.

Food consumption data from infants, toddlers, children, adolescents, adults and the elderly were used in the exposure assessment. For the present assessment, food consumption data were available from 41 different dietary surveys

[15]Extraction date 18 October 2024 from https://www.gnpd.com/sinatra/gnpd/search/#.

[16]Available online: https://www.efsa.europa.eu/en/data-report/food-consumption-data.

carried out in 22 EU Member States (Table 5). Not all Member States provided consumption information for all population groups, and in some cases the same country provided food consumption data from more than one consumption survey. In most cases, when, for one country and age class, different dietary surveys were available, only the most recent was used. However, when two national surveys from the same country gave a better coverage of the age range than using only the most recent one, both surveys were kept. For details on each survey, see Table A.4 of the Annex.

TABLE 5 Population groups considered for the exposure estimates of neotame (E 961).

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From more than 12 weeks up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia
Toddlers[a]	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, the Netherlands, Portugal, Slovenia, Spain
Children[b]	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, the Netherlands, Portugal, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, the Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, the Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
The elderly[b]	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, the Netherlands, Portugal, Romania, Slovenia, Spain, Sweden

[a]The term 'toddlers' in the Comprehensive Database (EFSA, 2011) corresponds to 'young children' in Regulations (EC) No 1333/2008 and (EU) No 609/2013.
[b]The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Comprehensive Database (EFSA, 2011).

Since 2018, all consumption records in the Comprehensive Database are codified according to the FoodEx2 classification system (EFSA, 2015). Nomenclature from the FoodEx2 classification system has been linked to the food categorisation system of Annex II of Regulation (EC) No 1333/2008, part D, to perform the exposure assessments. In practice, the FoodEx2 food codes were matched to the food categories. For a detailed description of the methodology used to link FoodEx2 codes to the food categories, see section 5.2.1 of EFSA FAF Panel (2024). In FoodEx2, facets are used to provide further information about different properties and aspects of foods recorded in the Comprehensive Database. Facets were used in the exposure assessment of neotame (E 961) to further identify foods to be included in the assessment (e.g. sweetener-related facets for foods in relevant food categories, see details in Annex A, Table A.2).

Food categories considered for the exposure assessment to neotame (E 961)

The food categories in which the use of neotame (E 961) is authorised were selected from the nomenclature of the Comprehensive Database (FoodEx2 classification system), at the most detailed level possible (up to FoodEx2 Level 7) (EFSA, 2015).

Facets were used to identify eating events referring to foods (including beverages) assumed to contain neotame (E 961) as well as foods related to specific restrictions defined in the legislation.

Facets were not used to identify relevant eating events for foods belonging to FC 05.3 Chewing gum, and for gum drops in FC 05.2 Other confectionery including breath refreshening microsweets, energy drinks in FC 14.1.4 Flavoured drinks and vitamin and mineral supplements in FC 17 Food supplements as defined in Directive 2002/46/EC excluding food supplements for infants and young children. These foods and food categories are expected to be major contributors to the exposure to sweeteners according to the literature and present a relatively high percentage of products labelled to contain at least one sweetener. Thus, all eating events referring to these foods and food categories were included in the exposure assessment as described in the protocol (EFSA FAF Panel, 2024).

As FC 17 Food supplements as defined in Directive 2002/46/EC excluding food supplements for infants and young children, does not consider food supplements for infants and toddlers as defined in the legislation, the exposure to neotame (E 961) from food supplements was not estimated for these two population groups.

FC 11.4 Tabletop sweeteners was not included in any of the scenarios as tabletop sweeteners are authorised at QS and no use levels or analytical data were submitted to EFSA.

Some restrictions/exceptions of certain food categories are not referenced in the Comprehensive Database and cannot be used to select specific foods. In that case, the whole food category was taken into consideration. In the case of regulatory maximum permitted level scenarios, when specific restrictions/exceptions defined in the legislation within the same food category could not be identified in the Comprehensive Database (see Section 3.2 the highest MPL among the authorised uses for the same food category was used for all, unless the highest value referred to a niche food. In that case, the next highest MPL was used.

Overall, 31 food categories with a quantified MPL were included in the *regulatory maximum level exposure scenario* (Table 4). Five food categories out of the 34 in which neotame is authorised were included in the brand loyal *refined scenario* based on available use level and analytical data and in the *refined regulatory maximum level exposure scenario*

based on available MPLs (FC 05.2 Other confectionery including breath refreshening microsweets, FC 05.3 Chewing gum, FC 14.1.3 Fruit nectars as defined by Directive 2001/112/EC and vegetable nectars and similar products, FC 14.1.4 Flavoured drinks and 17 Food supplements as defined in Directive 2002/46/EC (Protein and amino acids supplements only).

For FC 05.2 Other confectionery including breath refreshening microsweets one use level and three analytical results were available. As only three analytical results were available, the use levels of 50 mg/kg (mean) and 60 mg/kg (maximum) were used in the brand loyal refined scenario and considered more reliable by expert judgement. The use level of 50 mg/kg was equal to the highest analytical result submitted by Member States (see Tables A.1 and A.2 of the Annex).

The assigned concentrations to each food category in each exposure scenario are detailed in Table A.5 of the Annex.

3.4 | Exposure estimates

The Panel considered it appropriate, within the remits of the re-evaluation of sweeteners, to estimate a chronic exposure. As suggested by the EFSA Working Group on Food Consumption and Exposure (EFSA, 2011), dietary surveys covering only 1 day per subject were not considered as they are not adequate to assess repeated exposure. Similarly, subjects who participated only 1 day in the dietary studies, when the protocol prescribed more reporting days per individual, were also excluded for the chronic exposure assessment.

Exposure assessments of sweeteners under the re-evaluation programme are carried out by the FAF Panel based on two different sets of concentration data: (a) MPLs set down in the EU legislation (in the *regulatory maximum level exposure assessment scenario*) and (b) use levels and/or analytical data provided through calls for data (in the *refined exposure assessment scenario*).

To calculate chronic dietary exposure to neotame (E 961), food consumption and body weight data at the individual level were extracted from the Comprehensive Database and linked to the concentration data as described in section 5.2.1 of the Protocol (EFSA, 2024).

Chronic dietary exposures were calculated by combining concentration data of neotame (E 961) in each food with the average daily consumption for each food at individual level in each dietary survey and age class. Exposure estimates are divided by the individual's body weight resulting in a distribution of daily individual average exposures per kilogram body weight. On the basis of these distributions, the mean and 95th percentile (P95) exposures are calculated per survey and per age class. Mean estimates based on dietary surveys/age classes with less than six consumers and P95 estimated with less than 60 observations are not presented.

In this evaluation, as stated in section 5.2.3 in the Protocol (EFSA, 2024), the dietary exposure was assessed for only consumers of at least one food category containing neotame (E 961)[17] for all scenarios. Exposure estimates for these population groups are assumed to be the best approximate reflecting the exposure levels in diabetics, which is considered to be the population with the highest exposure to sweeteners (EFSA, 2024). Depending on the food categories considered in the exposure assessment, the exposure was estimated based on different numbers of consumers. Exposure estimates based on fewer food categories could be higher than those based on a larger number of food categories due to the higher number of non-consumers within certain food categories.

In order to evaluate if consumers-only of a single food category could have a higher exposure than consumers-only of at least one food category, the exposure to neotame (E 961) for consumers-only of each single food category (but still considering their whole diet) was also calculated for the *refined brand-loyal exposure assessment scenario*. These exposure estimates are discussed if they are higher than the exposure estimates for consumers only of at least one food category for this refined scenario.

Regulatory maximum level exposure assessment scenario

The *regulatory maximum level exposure assessment scenario* is based on the MPLs as set in Annex II to Regulation (EC) No 1333/2008 and in case of levels defined as *QS*, on the maximum reported use level/the highest reliable percentile of the analytical level when available. For neotame (E 961), the MPLs as listed in Table A.5 of the Annex were used. For the three food categories of FC 11.4 Tabletop sweetener, in which neotame (E 961) is authorised at *QS*, no use levels or analytical data were available. These food categories could therefore not be considered.

Refined brand-loyal exposure assessment scenario

The *refined brand-loyal exposure assessment scenario* for neotame (E 961) was based on two use levels reported by food industry and analytical data for three food categories provided by two Member States. This exposure scenario considers only those food categories for which these data were provided to EFSA. In the brand-loyal exposure assessment scenario,

[17]Part of the survey population may have no exposure to the additive because they did not report eating a food from one of the food categories that could contain the additive (these are 'non-consumers'). The sub-group who report eating these foods are called consumers.

it was assumed that a consumer is exposed long-term to neotame (E 961) present at the maximum reported use level of the analytical data for one food category and at the mean of typical use level/mean of analytical data for the other food categories. For more details, see the protocol (EFSA, 2024).

Concentration data used for the *refined brand-loyal exposure assessment scenario* are detailed in Table A.5 of the Annex.

Refined regulatory maximum level exposure assessment scenario

Results of the *regulatory maximum level exposure assessment scenario* are not comparable to the exposure estimates of the refined brand-loyal exposure assessment scenario. Since the number of food categories considered per scenario differs ($n = 31$ and 5, respectively), the underlying populations of consumers only are not the same. For this reason, the Panel also performed a *refined regulatory maximum level exposure assessment scenario* based on the same population group as included in the *refined brand-loyal exposure assessment scenario* (Table A.5 of the Annex).

3.4.1 | Dietary exposure to neotame (E 961)

Table 6 summarises the estimated exposure to neotame (E 961) from its use as a food additive in six population groups (Table 5) according to the *regulatory maximum level, the refined regulatory maximum level and the refined brand-loyal exposure assessment scenarios* for consumers only of at least one food containing the food additive.

TABLE 6 Summary of dietary exposure to neotame (E 961) from its use as a food additive in the regulatory maximum level, the refined regulatory maximum level and the refined brand-loyal exposure assessment scenarios, in six population groups among consumers only of at least one food category containing neotame (E 961) (minimum–maximum across the dietary surveys in mg/kg bw per day and number of corresponding dietary surveys in brackets)(c).

	Infants (12 weeks-11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (≥ 65 years)
Regulatory maximum level exposure assessment scenario[c]						
Mean[a]	0.01–0.11 (8)	0.01–0.26 (15)	0.01–0.18 (19)	0.01–0.10 (21)	0.01–0.06 (22)	< 0.01–0.03 (22)
95th perc[b]	0.02–0.20 (2)	0.03–0.95 (13)	0.05–0.61 (19)	0.03–0.34 (20)	0.02–0.22 (22)	0.01–0.12 (21)
Refined regulatory maximum level exposure assessment scenario[c]						
Mean[a]	0.02–0.32 (3)	0.01–0.34 (9)	0.02–0.22 (18)	0.02–0.11 (18)	0.01–0.09 (21)	0.01–0.09 (16)
95th perc[b]	– (0)	0.10–1.01 (3)	0.10–0.64 (12)	0.09–0.34 (12)	0.07–0.35 (15)	0.06–0.20 (7)
Refined brand-loyal exposure assessment scenario[c]						
Mean[a]	0.01–0.05 (3)	< 0.01–0.05 (9)	0.01–0.04 (18)	< 0.01–0.02 (18)	< 0.01–0.01 (21)	< 0.01–0.02 (16)
95th perc[b]	– (0)	0.06–0.16 (3)	0.02–0.12 (12)	0.01–0.07 (12)	0.01–0.05 (15)	0.01–0.03 (7)

[a]Mean estimates based on dietary surveys/population groups with less than six consumers may not represent the population group and are thus not included in this table.

[b]95th percentile estimates based on dietary surveys/population groups up to and including 59 observations may not be statistically robust (EFSA, 2011) and are thus not included in this table.

[c]Results of the regulatory maximum level exposure assessment scenario and the refined exposure assessment scenarios are not comparable as the underlying populations of consumers are different. This is due to a difference in the number of food categories considered ($n = 31$ and 5, respectively) and because facets are not considered in the regulatory maximum level exposure assessment scenario.

In the *regulatory maximum level exposure assessment scenario*, mean exposure to neotame (E 961) ranged from 0.01 mg/kg bw per day in all population groups to 0.26 mg/kg bw per day in toddlers. The 95th percentile of exposure ranged from 0.01 mg/kg bw per day in the elderly to 0.95 mg/kg bw per day in toddlers.

In the *refined regulatory maximum level exposure assessment scenario*, mean exposure to neotame (E 961) ranged from 0.01 mg/kg bw per day in toddlers and adults to 0.34 mg/kg bw per day in toddlers. The 95th percentile of exposure ranged from 0.06 mg/kg bw per day in the elderly to 1.01 mg/kg bw per day in toddlers.

In the *refined brand-loyal scenario exposure assessment scenario*, mean exposure to neotame (E 961) ranged from less than or equal to 0.01 mg/kg bw per day in all age groups to 0.05 mg/kg bw per day in infants and toddlers. The 95th percentile of exposure ranged from 0.01 mg/kg bw per day in adolescents, adults and the elderly to 0.16 mg/kg bw per day in toddlers.

Detailed results per population group and survey for the three scenarios are presented in Table A.6 of the Annex.

FC 14.1.4 Flavoured drinks was the main contributing category in all three scenarios for all population groups. More details on the contribution of each food category in the three scenarios, are available in Tables A7, A8 and A9 of the Annex.

The exposure estimates for consumers-only of each of the five food categories included in the refined scenarios, did not exceed the highest P95 exposure estimate in the *refined regulatory maximum level exposure assessment scenario* thus will not be further discussed.

Summary results for the additional scenarios are available in Table A.10 of the Annex

3.4.2 | Uncertainty analysis

In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2007), the following sources of uncertainties were considered and summarised in Table 7.

TABLE 7 Qualitative evaluation of influence of uncertainties on the dietary exposure estimate.

Sources of uncertainties	Direction[a]
Consumption data	
Different methodologies/representativeness/underreporting/misreporting/no portion size standard/only a few days	+/–
Underreporting of food descriptors (facets) concerning the presence or potential presence of sweeteners	–[b]
Not considering some of the restrictions specified in the legislation (e.g. flavour enhancer only)	+/–
Concentration data	
Correspondence of reported use levels and analytical data to the food items in the Comprehensive Database: uncertainties to which types of food the levels refer	+/–
Uncertainty in possible national differences in use levels of food categories	+/–
Refined regulatory maximum level exposure assessment and brand-loyal scenario: Only five food categories for which use levels/analytical results were available were included out of 34 food categories in which neotame (E 961) is authorised	–[b]
Use levels considered applicable to all foods within the entire food category, while the percentage of the foods in a subcategory labelled with neotame (E 961) in Mintel was maximally 2.8%	+
Refined scenarios: Some food categories for which some products were available in Mintel were not included as no concentration data were available (e.g. sushi)	–[b]
Methodology	
Refined regulatory maximum level scenario: exposure calculations based on the MPLs according to Annex II to Regulation (EC) No 1333/2008	+
Refined brand-loyal scenario: exposure calculations based on the mean and maximum (or highest reliable percentile) levels	+/–
Use of data from food consumption survey covering only a few days to estimate high percentile(95th) of long-term (chronic) exposure	+

[a]+, uncertainty with potential to cause overestimation of exposure; –, uncertainty with potential to cause underestimation of exposure.
[b]Direction of the uncertainty is based on the assumption that the underlying population of consumers does not change.

Overall, the Panel considered the exposure to neotame (E 961) from its use as a food additive in European countries present in the Comprehensive Database to be an overestimation in all scenarios considering the very limited number of products containing neotame (E 961) in Mintel's GNPD (about 0.07% of products in the categories in which at least one product was labelled to contain neotame (E 961), see Section 3.3.2).

The *refined brand-loyal scenario* is considered by the Panel the most appropriate scenario for the risk assessment of the current dietary exposure as it is based on use levels and analytical data related to foods available on the market.

3.4.3 | Concentrations and dietary exposure data reported for neotame (E 961) in the EU literature

A literature search was carried out to collect data on the levels of neotame (E 961) in food and dietary exposure data to this sweetener. Bibliographic searches were conducted in bibliographic databases or scientific citation search platforms (see Appendix B). Several published articles were retrieved and reviewed.

From these, relevant articles were selected when neotame (E 961) was analysed in foods (including beverages) representative for European food markets and/or when an assessment of dietary exposure to neotame (E 961) for the European population was performed. From the four relevant articles related to the determination of concentration levels of artificial sweeteners in foods, neotame (E 961) was never detected in any of the analysed samples from all reported studies:

1. 25 different samples of beverage obtained from local supermarkets in Spain including soft drinks of different flavours (cola, orange, etc..) with or without gas, nectars and mixed drinks containing beer or milk were analysed (Ordoñez et al., 2015).
2. 66 beverage products commonly consumed and available on the Spanish market from national and international industries that include energy drinks, soft drinks, juices, dairy based drinks, soy drinks, teas, beers and spirit alcoholic drinks were analysed (Lorenzo et al., 2015).
3. 290 samples representative from large and small holding companies of the Italian market for all foods categories in which sweeteners are allowed by EU-legislation, including drinks, jams, yoghurts, ice creams, confectionery, tabletop sweeteners and food supplements, were collected during the spring of 2014 in Roma and analysed (Janvier et al., 2015).

4. 179 samples of different brands of soft drinks, beverages, juices, yoghurts, jams, marmalades, canned fruits, vegetable salads, preserved vegetables and fish products available on the Polish market from local supermarkets and retail stores in Gdansk were collected from June 2010 to January 2011 and analysed (Zygler et al., 2012).

Le Donne et al. (2017) and Dewinter et al. (2016) estimated the dietary exposure to neotame (E 961) using a tiered approach in Italian and in Belgium population groups, respectively.

1. Based on food consumption data of a study sample of 3270 Italian individuals over the age of 3 years combined with the corresponding EU-MPLs, dietary exposure was estimated at 0.03 mg/kg bw/day at the average level and 0.13 mg/kg bw/day at the 95th percentile (Le Donne et al., 2017). No assessment of the dietary exposure to neotame (E 961) was performed at the next steps of the tiered approaches because from the chemical analysis of Italian products from Lorenzo et al. (2015), neotame (E 961) was not detected in any of the samples, a finding which was confirmed by a food label survey showing that no products contained neotame (E 961) as added sweetener according to the information on the label.
2. Based on a diary survey conducted in children with type 1 diabetes mellitus aged 1–18 years ($n = 242$), estimates of the consumption of most food categories in which non-nutritive sweeteners are used were combined with the corresponding EU-MPLs (Dewinter et al., 2016). Resulting average dietary exposure estimates ranged from 0.15 mg/kg bw/day in 13–18 years old population to 0.27 mg/kg bw/day in 4–6 years population. At the P95 of consumers only, exposure to neotame (E 916) ranged from 0.59 to 1.03 mg/kg bw/day in the same population groups. The second tiered approach step using the reported uses level provided by a recent Belgium study was not performed as no use levels were available for neotame (E 961).

Despite differences in methodologies and data sources, concentrations in foods and the dietary exposure estimates reported in the EU literature were consistent with the data and exposure assessment results in the current assessment. The literature also confirms that neotame (E 961) is a sweetener that is rarely used in foods on the European market.

3.5 | Biological and toxicological data

No new data were received in response to the call for biological and toxicological data.[6]

From the literature search performed,[18] the new studies which were considered as relevant for this risk assessment, according to the inclusion criteria reported in the protocol on hazard identification and characterisation (EFSA, 2020a; EFSA FAF Panel, 2023), were: one study on the efficacy of neotame addition to animal feed in pigs (Zhu et al., 2016); one study on taste preference behaviour in mice (Yin et al., 2020); one study in humans (Gibbons et al., 2024). These studies are reported in Sections 3.5.6, 3.5.7 or Appendix B.

3.5.1 | Absorption, distribution, metabolism and excretion

No new data were received following the call for biological and toxicological data,[6] and no new data were identified from the literature search. However, as described below, new data were provided upon specific request by EFSA in relation to the genotoxicity assessment (see Section 3.5.2).

In the previous evaluation of neotame (E 961) by EFSA (2007), the AFC Panel reviewed studies on ADME available at that time. Those unpublished studies were part of the original submission of an application on neotame as a new food additive and they are reported in Appendix B of the EFSA 2007 opinion. A summary of these studies is presented in this section. More details can be found in the 2007 EFSA opinion.

Rats (three males and three females per dose group) were exposed to ^{14}C-radiolabelled neotame (labelled in the C1 position of the 3,3-dimethylbutylamine moiety) by oral gavage at single doses of 15 and 120 mg/kg bw and intravenously at 15 mg/kg bw (cited in EFSA, 2007 as PCR 1027). After oral gavage of labelled neotame, the peak plasma radioactivity was observed around 30 minu after administration. However, intact neotame was only detected in the plasma of 3 of the 6 animals orally dosed at 120 mg/kg bw (at levels just above the limit of quantitation). The comparison between the plasma radioactivity AUCs for oral and intravenous administration indicates that the oral bioavailability of total radioactivity was approximately 20%, and was similar in males and females.

Dogs (three males and three females per dose group) were exposed to ^{14}C-radiolabelled neotame (labelled in the C1 position of the 3,3-dimethylbutylamine moiety) by oral gavage at single doses of 15 and 120 mg/kg bw and intravenously at 15 mg/kg bw (cited in EFSA, 2007 as PCR 1029). After oral gavage of labelled neotame, the peak plasma radioactivity was observed within 30 mins after administration in both males and females. The authors estimated that 33% and 47% of the radioactivity was absorbed after 15 and 120 mg/kg bw respectively (based on the amount of radioactivity excreted in urine following oral versus intravenous administration). Between 1% and 6% of the oral dose was found to be intact neotame

[18]Timespan applied to search strategy covers the period since the latest EFSA evaluation (i.e. 2007) with an overlap of 1 year (i.e. 2006) until 2 October 2024.

in the urine, with the remainder as the metabolite NC-00751 (de-esterified neotame) with 3 other minor metabolites. No intact neotame was found in the faeces after oral dosing. The metabolite NC-00751 accounted for the radioactivity in faeces (62%–74% of oral dose). The FAF Panel considered that this indicates a substantial biliary excretion of the de-esterified neotame and/or intestinal metabolism of unabsorbed neotame.

In a human study, seven healthy male volunteers ingested ^{14}C-radiolabelled neotame (labelled in the C1 position of the 3.3-dimethylbutylamine moiety) at a single dose of 18.75 mg (approx. 0.25 mg/kg bw) after an overnight fast. One subject was separately used to determine optimal times for collection of urine and faeces (cited in EFSA, 2007 as PCR 1039). Radioactivity was rapidly absorbed (peak plasma radioactivity observed within approximately 30 min after administration) and excreted in urine and faeces, with a mean recovery of 98% of radioactivity within 72 h of ingestion. Based on the urinary excretion of radioactivity, at least 34.3% was absorbed and systemically available following oral administration.

In the first safety evaluation of neotame (E 961) by EFSA (2007) as a basis for its authorisation as a new food additive, the metabolism and pharmacokinetics of neotame were also examined in mice and rabbits. The data provided by the IBO at that time demonstrated that neotame was rapidly but incompletely absorbed in all species (List of studies provided in Appendix B on page 39 of the EFSA, 2007 opinion).

Figure 4 shows the metabolic pathway of neotame (as adapted from JECFA, 2004).

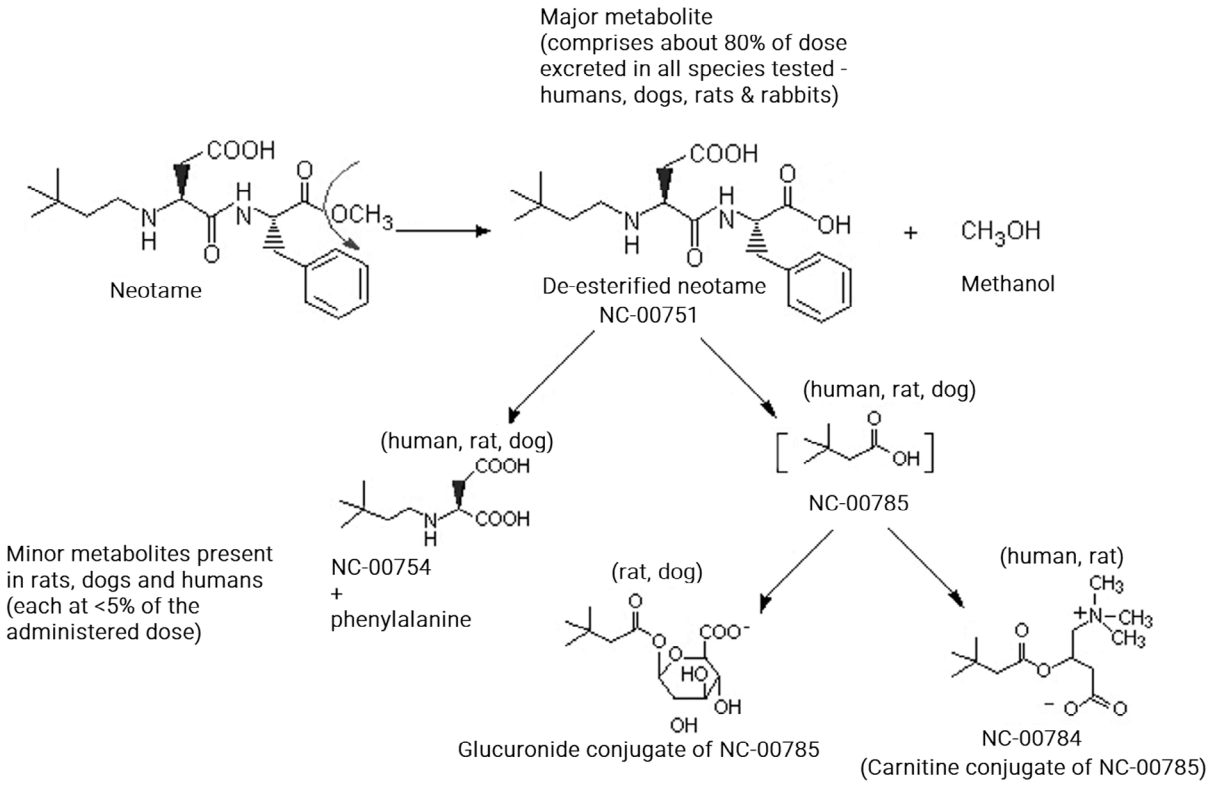


FIGURE 4 Metabolism of neotame (as adapted from JECFA, 2004).

Data on the validation for the determination of neotame and the metabolite NC-00751 in mouse plasma using liquid chromatography with tandem mass spectrometric detection were provided in response to a call for genotoxicity data[19] (Documentation provided to EFSA, n. 11). The method described was considered suitable for the determination of neotame and NC-00751 in treated mouse plasma over the calibration range of 0.5–50.0 ng/mL, with a limit of quantification (LOQ) of 0.5 ng/mL. Acceptable accuracy (recovery), precision (repeatability), linearity (calibration) and specificity (interference) were observed over the concentration range 0.500–50.0 ng/mL using a sample volume of 20 µL. Using this methodology, data were submitted on the plasma concentrations of neotame and the metabolite NC-00751 in male and female mice following a single administration of neotame by gavage (Documentation provided to EFSA, n. 10). A single oral administration of neotame NC-723 at doses of 500, 1000 and 2000 mg/kg bw to groups of six in male and six female mice did not result in any abnormal clinical signs. The concentration of neotame in plasma samples collected 24 and 48 h after treatment were below the LOQ (1.5 ng/mL) in 33 out of 36 treated animals. Low, but quantifiable concentrations of the unchanged substance were detected in 3 male mice, one (M6) dosed with 1000 mg/kg (1.90 ng/mL at time point 48 h) and two dosed with 2000 mg/kg (2.04, at time point 24 h in M8 and 3.34 ng/mL, at time point 48 h in M7). In contrast, the metabolite NC-00751 was detected in plasma samples of most (31 out of 36) treated animals. NC-00751 plasma levels were highly variable and

[19] https://www.efsa.europa.eu/en/call/call-data-genotoxicity-data-sweeteners.

inconsistent between animals and sex, with no apparent relation with the administered dose: from < LOQ (1.5 ng/mL) to 348 ng/mL (low dose), from < LOQ to 53.6 ng/mL (middle dose) and from 1.57 to 93.1 ng/mL (high dose) in both sexes. These data indicate that neotame was absorbed, rapidly and extensively metabolised, and that there is systemic exposure to the metabolite NC-00751. Due to the selection of the sampling time points and because of the extensive in vivo biotransformation of neotame to NC-00751, the parent compound was not found in blood samples of most of the animals.

The FAF Panel considered that neotame is rapidly absorbed and pre-systemically metabolised in both animals and humans. Any systemic intact neotame is likely excreted in the urine along with its metabolites. A significant fraction of neotame metabolites may result from pre-systemic metabolism. The data are consistent with biliary excretion in dogs. The Panel noted that the major degradation product N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751) is also the primary metabolite of neotame, which implies that the available toxicological dataset also informs on the toxicological profile of NC-00751, being an impurity, a degradation product and a metabolite. The Panel noted that NC00751 is formed by de-esterification resulting in a molecule which is structurally similar to the parent compound.

3.5.2 | Genotoxicity

No new data were received following the call for biological and toxicological data,[6] and no new data were identified from the literature search. As described below, new data was only provided upon specific request by EFSA.

In the previous evaluation of neotame (E 961) by EFSA prior to its authorisation for use, the AFC Panel reviewed the available in vitro and in vivo genotoxicity studies on neotame (cited in EFSA, 2007 as PCR 0963, PCR 0964, PCR 0965) and its metabolites/degradation products. Based on the negative results obtained in gene mutation assays in bacteria and in mammalian cells, in in vitro chromosomal aberration assay and in in vivo micronucleus test in mice, the AFC Panel concluded that neotame was of no concern with respect to genotoxicity.

The major degradation product/metabolite NC-00751 as well as three minor degradation products (NC-00764, NC-00777 and NC-00779, see Figure 3) were also considered by the AFC Panel to be of no genotoxicity concern based on the results of gene mutation assays in bacteria and mammalian cells and in vivo micronucleus tests (cited in EFSA, 2007 as study PCR 1086, PCR 1087, PCR 1090, PCR 1137, PCR 1138, PCR 1191, PCR 1192, PCR 1196PCR 1201, PCR 1202, PCR 1206).

The hypothetical formation of nitrosamines in the gastrointestinal tract from the reaction of nitrite with neotame and its major degradation product/metabolite NC-00751 was also considered in the previous evaluation (EFSA, 2007). Under simulated gastric conditions, no nitrosated neotame NC-00799 (N-nitroso-(3,3-dimethyl)-L-aspartyl-L-phenylalanine methyl ester, N-nitrosoneotame;) and no nitrosated de-esterified neotame NC- 00800 (N-nitroso-(3,3-dimethylbuthyl)-L-aspartyl-L-phenylalanine,) were detected. Moreover, these two nitrosated products (from NC-00799 and NC- 00800) were evaluated as not mutagenic in the Ames test, using a protocol optimised for the detection of genotoxic nitrosamines (cited in EFSA, 2007 as 'NC-00799 and NC-00800 bacterial mutation test'. Final report. Charles River Laboratories. August 2007). The AFC Panel considered that the information available was sufficient to conclude that any possible nitrosation of neotame, should it occur, was of no concern with respect to genotoxicity.

The FAF Panel noted that the in vitro gene mutation and chromosomal aberration studies in mammalian cells previously evaluated by the AFC Panel, although not fully aligned with the most recent version of the relevant OECD guidelines (OECD TG473, 2016a and OECD TG490, 2016b) are sufficiently reliable to be considered in this re-evaluation. The FAF Panel also noted that an in vitro micronucleus assay was not part of the data set assessed in the previous EFSA opinion on neotame (EFSA, 2007). The dataset available for assessment at that time was in line with the previous SCF/EFSA recommendations (which consisted of the Ames test and the in vitro gene mutation and chromosomal aberration assays). However, the subsequently adopted guidelines on genotoxicity testing strategy (EFSA Scientific Committee, 2011) introduced the requirement for an in vitro micronucleus assay to include the assessment of aneugenicity (numerical chromosomal aberrations) in addition to clastogenicity.

The FAF Panel also noted that no information on polyploidy, as a surrogate indicator of aneugenic potential, was included in the previous in vitro chromosomal aberration study on neotame (cited in EFSA, 2007 as PCR 0964). Moreover, the FAF Panel considered that the lack of an in vitro micronucleus assay on neotame was not addressed by the available in vivo micronucleus test (cited in EFSA, 2007 as PCR 1026). In fact, according to current guidelines, the in vivo study was considered inconclusive in view of the lack of data demonstrating bone marrow exposure under the experimental conditions of the test.

Upon request from EFSA, a new in vitro micronucleus test was subsequently submitted by one interested party (Documentation provided to EFSA N. 5, 2021). The study was performed in compliance with GLP, following the most recent available version of the OECD TG for the in vitro micronucleus test (OECD TG 487, 2014-2016). The test item neotame (purity 99.2%) was tested in duplicate cultures of human lymphocytes at three concentrations using a 4 h exposure in the presence and absence of metabolic activation, and at four concentrations using a 24 h exposure in the absence of metabolic activation. Vehicle (DMSO) and positive controls were concurrently tested using quadruplicate and duplicate cultures, respectively. The concentration levels evaluated were selected based on the cytokinesis block proliferation index (CBPI) induced by treatment (determined in a preliminary toxicity test) with the aim to achieve the target toxicity of 55 ± 5%, as indicated in the OECD 487 guideline. The following concentrations were tested: 0, 500, 1000 and 1200 µg/mL using the 4 h exposure (with and without metabolic activation), and 250, 350, 400 and 450 µg/mL using the 24 h exposure only in the absence of metabolic activation. At the end of the exposure period, the cell cultures were washed and then incubated

for a further 24 h in the presence of Cytochalasin B. After 4 h exposure to the test substance, a statistically significant and concentration-related increase in the frequency of binucleated cells with micronuclei was observed both in the absence ($p < 0.001$) and in the presence ($p < 0.01$) of metabolic activation. Cytotoxicity did not exceed the recommended target level (45% and 46% at the highest dose level without and with S9, respectively). Also, after 24 h exposure in the absence of metabolic activation, the frequency of binucleated cells with micronuclei was reported to be significantly elevated at all concentrations ($p < 0.05$ for the lowest concentration tested; $p < 0.001$ all subsequent concentrations) with a significant trend test ($p < 0.001$). Cytotoxicity ranged from 15% to 57% at the highest concentration. Vehicle and positive controls displayed frequencies of cells with micronuclei within the historical control limits.

The Panel concluded that neotame has the capacity to induce the formation of micronuclei in a mammalian cell system in vitro, in the presence and in the absence of metabolic activation. The data available from this study did not allow distinguishing whether micronuclei arise from clastogenic or aneugenic events, or both. The Panel noted that the available in vivo micronucleus assay in mice (cited in EFSA, 2007 as PCR 1026) did not provide sufficient information to assess whether the effects observed in vitro are expressed in vivo, as no exposure of the target tissue (i.e. bone marrow) to the test item was demonstrated in that study or in toxicokinetic studies with oral administration of neotame in mice. The Panel concluded that evidence should be provided for bone marrow exposure under the identical conditions to the mouse in vivo micronucleus assay. The use of data from rats as evidence of exposure would only be acceptable if the conditions of the study were identical to the mouse study and if a rationale (substantiated by data) were provided.

In order to collect the information required to complete the genotoxicity assessment of neotame (E 961) and other sweeteners, a call for data was published by EFSA.[20] In response to this call, data were submitted to EFSA demonstrating that neotame was absorbed and that systemic exposure to the metabolite NC-00751 occurred under the conditions of the in vivo bone marrow mouse MN assay (see ADME Section 3.5.1).

Based on the new evidence for systemic exposure to NC-00751, the in vivo MN test result is considered by the Panel to be sufficient to rule out genotoxic effects due to systemic exposure to neotame via oral intake. However, the data available do not overcome a safety concern for the possible effects of neotame, the parent compound, at the site of contact, where the concentrations will be maximal, bearing in mind that the positive findings in the in vitro MN assay were seen also in the absence of metabolic activation. Consequently, the potential genotoxic effects at the site of contact (gastrointestinal tract) remained not sufficiently addressed.

Considering the available experimental data, the Panel noted the negative results of chromosomal aberrations assay, as well as QSAR analyses that gave no alerts for DNA reactivity and genotoxicity (OECD QSAR toolbox, Appendices D and F). Consequently, the Panel considered that the positive findings in the in vitro MN assay could be due to aneugenicity.

As validated test methods to evaluate in vivo aneugenic effects in the GIT are not yet available, following the recommendations of the EFSA SC Guidance on Aneugenicity Assessment (EFSA Scientific Committee, 2021a), as a pragmatic approach, the Panel compared the estimated concentration associated with an aneugenic effect in the available in vitro micronucleus test in human lymphocytes with the concentration of neotame estimated to be present in the GIT following ingestion.

Data on micronucleus induction in vitro were modelled applying a benchmark dose (BMD) approach in order to identify a reference point for aneugenicity assessment. The BMD analysis was performed using the EFSA 'Bayesian BMD' webtool. The EFSA guidance on BMD modelling (EFSA Scientific Committee, 2022) does not provide formal guidance on the modelling of data derived from in vitro micronucleus (MN) assays, in particular the selection of the appropriate benchmark response (BMR). Taking into account information provided in relevant scientific literature, OECD guidance documents and expert judgement, the FAF Panel re-analysed the data provided by the IBO (Documentation provided to EFSA No. 4) to derive a BMD. A detailed description of the data analyses performed is provided in Appendix G and related Annexes.

In brief, the FAF panel converted the reported data from %MN to the frequency of binucleated cells (number of MN observed per 1000 cells), a binary variable which can be analysed using models for quantal data. The FAF Panel used historical negative control data reported by the IBO (Documentation provided to EFSA No. 4) for deriving a BMR for the 4 h MN assay (without metabolic activation) and 24 h MN assay, respectively. The Panel decided to use BMRs corresponding to the 95th quantile (from the estimated Beta distribution based on the historical control data reported), as in the OECD TG487 it is recommended that concurrent negative controls should ideally be within the 95th percentile control limit. Considering that the data used for this analysis were concentrations and not doses, the Panel considered it appropriate to use the term benchmark concentration (BMC), rather than BMD.

For the 4 h MN assay (without metabolic activation), the BMC was 200 mg/L (BMCL 44, BMCU 365 mg/L). With respect to the 24 h MN assay, the BMC was 211 mg/L (BMCL 156, BMCU 250 mg/L) . As a worst-case scenario for exposure, because of only minimal dilution in the oesophagus, the Panel considered a concentration equal to the currently authorised maximum permitted level (MPL) for beverages which is 20 mg/L (see Table 4). The Panel noted however that the maximum reported use levels or analytical concentration in beverages are lower (7 mg/L for FC 14.1.3 and 2.8 mg/L for FC 14.1.4, respectively). The Panel is aware that higher MPLs are established in other food categories (e.g. chewing gum, microsweets) for which dilution during ingestion would lower the neotame concentration below the concentration assumed for beverages.

In the Guidance on Aneugenicity (EFSA Scientific Committee, 2021), it is stated that 'if the concentration in the oesophagus/stomach is estimated to be of the same order of magnitude as the concentrations shown to be aneugenic *in vitro*, there

[20] https://www.efsa.europa.eu/en/call/call-data-genotoxicity-data-sweeteners.

is concern for aneugenicity in vivo'. Because of the relatively short contact time in the oesophagus, the 4 h data should mimic the consumer exposure closer than the 24 h data, however the 24 h data were selected since they represents the more severe treatment conditions to elicit an aneugenic effect compared to the 4 h timepoint. Furthermore, it allows for the estimation of the BMC with a higher precision given the concentration ranges tested, i.e. lower active concentration ranges used in this experiment. Therefore, the Panel selected the BMCL value of 156 mg/L estimated from the 24 h data for comparison to the concentration in the beverages. This value is approximately 8-fold higher than the MPL of 20 mg/L for beverages. Comparing the reported use levels and analytical data in beverages with the BMCL would result in a difference of more than an order of magnitude.

Since, for neotame, the toxicological dataset available is not limited only to genotoxicity data, evidence from the available toxicological data was also considered by the Panel, as recommended in the EFSA SC statement 'Clarification of some aspects related to genotoxicity assessment' (EFSA SC, 2017). Such evidence includes lack of any indication for carcinogenicity in two studies in mice and rats with doses up to 1000 mg/kg bw per day and the lack of adverse effects, including lack of histopathological effects in the GIT (see Section 3.5.4).

Overall, taking into account all the evidence available, the Panel considered that there is no concern for genotoxicity of neotame (E 961) at the MPL as well as at the reported use levels and concentrations measured in beverages.

In silico assessment of neotame (E961) degradation products/impurities

The results of an in silico assessment of the genotoxicity of the neotame impurities listed in Appendix D was performed using a suite of VEGA statistical models and the OECD QSAR Toolbox. The results are reported in Appendix F and the main findings are summarised here below.

The QSAR analysis by VEGA models did not identify relevant alerts for mutagenicity in the Ames test, chromosomal aberrations and activity in the in vivo micronucleus test in nine of the 14 compounds analysed (1–4 and 7–11). The only positive prediction, common to all analysed compounds, including phenylalanine (phe), was for the activity in the in vitro micronucleus test. The pattern of VEGA predictions is consistent with the observed mutagenicity profile of neotame E961, which was negative in all assays apart from the in vitro micronucleus test. As the same alerting fragments for the activity in the in vitro micronucleus tests were identified in the molecular structure of neotame and its impurities, the safety assessment of these substances is covered by the safety assessment of neotame E961.

In the same compounds (1 to 4 ad 7 to 11), the OECD QSAR Toolbox profilers did not identify alerts for mutagenicity or DNA binding, except for the generic flagging of the possible metabolic formation of quinone species due to the presence of a benzene ring. This alert can be considered too generic and not predictive for this set of compounds, as it was also found in substances (NC-00764, NC-00777 andNC-00779, in addition to Neotame E961) which tested negative in genotoxicity assays detecting DNA damaging substances (Ames test, in vitro mammalian gene mutation and chromosomal aberration tests).

For compounds 5 and 6, the presence of an alpha-beta unsaturated carbonyl group was identified as an alert for potential mutagenicity in the Ames test by one Toolbox profiler. This alert is reported to have a low Positive Predictivity (0.31) for Ames mutagens (Benigni et al., 2008). To evaluate the relevance of this alert in compounds 5 and 6, a read across analysis was performed searching analogues with experimental data in the genotoxicity Toolbox databases. The search was focused on chemicals with the alert embedded in the same molecular environment of the two compounds (alert vicinal to carboxylic acid moiety). One sufficiently similar chemical was found (Penicillic acid, CAS 90-65-3) in the genotoxicity Toolbox databases. Penicillic acid is reported to be negative in the Ames test (strains TA100, TA1538, TA1535, TA98, TA37; with and without S9 mix).

For the same compounds, the consensus of several QSAR models in the VEGA platform was for the lack of mutagenicity in the Ames test. A prediction of activity in the chromosomal aberration test in vitro and, for compound 5 only, in the in vivo micronucleus test was made by other QSAR models in the VEGA platform. However, these predictions were considered unreliable as the training set did not include compounds closely related to compounds 5 and 6. Overall, no reliable indication of potential genotoxicity of compounds 5 and 6 was provided by the integrated application of several QSAR models.

Both neotame nitrosated products (compounds 12–13) were flagged as potentially genotoxic by the QSAR Toolbox profilers due to the presence of an alkyl N-nitroso group. However, no mutagenic activity was predicted for these nitrosated derivatives by most VEGA models, in agreement with the lack of mutagenicity in the Ames tests of both compounds (NC-00799 and NC-00800) reported in the previous evaluation of the EFSA AFC Panel in 2007 (EFSA, 2007). Moreover, these molecules were not detected under simulated gastric conditions, and thus they can be considered of no concern with respect to genotoxicity of neotame E961 (see Section 3.5.1).

Overall, the Panel considered that there is no concern for genotoxicity of impurities and degradation products of neotame (E 961).

3.5.3 | Acute toxicity

No new data were received following the call for biological and toxicological data,[6] and no new data were identified from the literature search. No acute toxicity studies on neotame were reported in the previous EFSA opinion on neotame, which however presents the results of the investigation of acute toxicity of three of its minor degradation products in

Sprague–Dawley rats administered by oral gavage in doses up to 3.0 mg/kg bw (NC-00779) or to 6.0 mg/kg bw (NC-00764, NC-00777) (as reported in EFSA 2007) and observation for 14 days. The results did not show acute toxic effects of these three degradation products at these low doses (EFSA, 2007). The Panel noted that these data are of interest concerning the toxicity testing of the minor degradation products; however, since an acute reference dose for the food additive is not needed, no further information with higher doses is necessary.

3.5.4 | Short-term and sub-chronic toxicity

No new data were received following the call for biological and toxicological data,[6] and no new data were identified from the literature search. In the evaluation of neotame (E 961) by EFSA (2007), the AFC Panel reviewed short-term and sub-chronic toxicity studies available at that time. These unpublished studies were part of the original submission of an application on neotame as a new food additive and they are reported in Appendix B of the previous EFSA (2007) opinion. A summary of these studies is presented in this section. More details can be found in the 2007 EFSA opinion.

In one 13-week range-finding study, neotame was administered to mice via diet (20 animals/sex/group) at doses of 0, 100, 1000, 4000 or 8000 mg/kg bw per day (cited in EFSA, 2007 as PCR 0989). The AFC Panel considered NOEL to be 1000 mg/kg bw, based on the statistically significantly increases on relative liver weight at 4000 and 8000 mg/kg bw in both sexes, which were not accompanied by microscopical changes.

In a 13-week dietary toxicity study in rats (20 animals/sex/group), neotame was administered at doses of 0, 100, 300, 1000 or 3000 mg/kg bw per day (cited in EFSA 2007 as PCR 0988). The AFC Panel considered NOEL to be 300 mg/kg bw based on the slight but statistically significant increases in alkaline phosphatase (AP), observed in the 1000 and 3000 mg/kg bw groups (10%–20% higher compared to controls, respectively).

In beagle dogs, a 13-week dietary toxicity study was followed by a 4-week reversibility period, and neotame was administered to five animals/sex/group at doses of 0, 60, 200, 600 or 2000 mg/kg bw (cited in EFSA, 2007 as PCR 0990). The AFC Panel considered the NOEL to be 200 mg/kg bw based on the statistically significant increase activity of serum AP of dogs at doses of 600 and 1200 mg/kg bw in both sexes after 6 and 13 weeks. The increases in serum AP activity at the high dose were less than four-fold above the control values and were approximately 2.5 times the pre-dosing values. Serum AP activity returned to normal levels in both males and females during the 4-week recovery period.

In the three studies described above, the highest doses of neotame were associated with a reduced body weight/body weight gain and reduced feed intake. This was observed at a dose of 3000 mg neotame/kg bw per day in the 13-week study in rats and at a dose of 2000 mg/kg bw per day in the 13-week study in dogs. In a 13-week study in mice, a decreased body weight relative to control was recorded at neotame doses of 4000 and 8000 mg/kg bw per day and was associated with increased feed scattering. The changes in body weight respective to controls were considered by the ACF Panel as not adverse or indicative of toxicity but a consequence of the decrease of feed intake due to reduced palatability of the neotame- containing diets, and therefore the body weight changes were not considered as appropriate for identification of the NOAELs.

Two palatability/diet preference studies were conducted in rats receiving neotame in the diets for 14 days, at concentrations ranging from 50 up to 15,000 mg/kg in the diet (equivalent to 6 to 1763 mg/kg bw) (cited in EFSA, 2007 as PCR 1132 and 1150). Results showed that neotame decreased the palatability of diets even at relatively low concentrations of 50 to 150 mg/kg in diet (equivalent to 6 to 18 mg/kg bw per day) or greater.

The increase in serum AP activity in rats and dogs, with a NOEL of 300 and 200 mg/kg bw/day, respectively, was considered as a pivotal effect by the ACF Panel (cited in EFSA, 2007 as PCR 0988 and PCR 0990). The AFC Panel further concluded that no- treatment- related adverse effects were observed in a 4-week dietary study in rats with a mixture of the three minor neotame metabolites (NC-00764, NC-00777 and NC-00779) (cited in EFSA, 2007 as PCR 1186). The FAF Panel agreed with the conclusions from 2007 by the AFC Panel.

In a 52-week toxicity study in beagle dogs, neotame was administered via the diet at doses of 20, 60, 200 or 800 mg/kg bw, followed by a 4-week reversibility period, in which animals in all groups were fed a standard diet (cited in EFSA, 2007 as PCR 1017). Control and the two highest dose groups consisted of six animals/sex/group while the other two groups had four animals/sex/group. The additional two dogs/sex in the control and in the two highest dose level groups were used for the reversibility phase of the study. Feed consumption was reduced for males at 800 mg/kg bw during the first 2 weeks and it was lower, but not statistically significantly, until week 4. Body weight gains were also decreased, although not significantly, for males at 800 mg/kg bw during the first 8 weeks of the study. The only clinical observation considered to be treatment-related was 'grey faeces' at 800 mg/kg bw, which was not observed after the first day of the reversibility period. No treatment-related effects were reported on haematology or on organ weights, or any gross or microscopic findings were observed. There was no indication of organ toxicity, immunotoxicity or neurotoxicity. The transient decreases in feed intake (until week 4) and in body weight gain (until week 8) in males receiving the neotame dose of 800 mg/kg bw per day were considered by the AFC Panel as related to a reduced palatability of diet containing neotame and not to toxicity of the compound. The only consistent and treatment-related effect was a statistically significantly increased serum AP activity at 800 mg/kg bw in both sexes at all time points tested (weeks 13, 26, 39, 52). This effect was reversible when animals have returned to a basal (control) diet following 52 weeks of treatment (4-week reversibility period). Isozyme analysis demonstrated that the increase in serum AP activity was due to elevation of the hepatic AP isoenzyme. There was a four–six-fold increase in the liver AP isoenzyme when compared to controls. There was no statistically significant increase in either the

corticosteroid-induced AP isoenzyme or the bone AP isoenzyme. The elevation of AP (activity was not accompanied by any microscopic evidence of cholestasis or hepatotoxicity, lesions in intestines or bone, or other clinical chemistry evidence of effects on the hepatobiliary system. The AFC Panel considered the increase in AP (activity to be the critical endpoint and identified a NOAEL of 200 mg neotame/kg bw per day from the 52-week study in dogs. The AFC Panel derived an ADI of 2 mg/kg bw based on the application of a 100-fold safety factor (EFSA, 2007).

3.5.5 | Chronic toxicity and carcinogenicity

No new data were received following the call for biological and toxicological data,[6] and no new data were identified from the literature search.

In the evaluation of neotame (E 961) by EFSA (EFSA, 2007), the AFC Panel reviewed both long-term studies in rats and dogs as well as carcinogenicity studies in mice and rats, available at that time. These unpublished studies were part of the original submission of an application on neotame as a new food additive and they are reported in Appendix B of the previous EFSA 2007 opinion. A summary of these studies is presented in this section. More details can be found in the 2007 EFSA opinion.

In a 52-week toxicity study with exposure in utero and followed by a 4-week reversibility period, a parent generation (F0) (25 animals/sex/group) of Sprague–Dawley rats,, received neotame via the diet at concentrations to provide doses of 0, 10, 30, 100, 300 and 1000 mg/kg bw/day, for 4 weeks before and throughout pairing (cited in EFSA, 2007 as PCR 1011). Females from the F0 generation continued treatment throughout gestation and lactation to weaning at postnatal day (PND) 21. After weaning, one male and one female from each litter were chosen for a 52-week toxicity study. Groups consisted of 20 animals of each sex and were exposed to the same dose levels as the parental generation. The effects on feed consumption and body weight (at the three highest doses) were considered to be linked to the reduced palatability of diet containing neotame and not to toxicity of the compound. The Panel noted that there were no effects reported on AP in this study.

In a carcinogenicity study CD-1 mice (70/sex/group) were administered neotame at dietary concentrations providing doses of 50, 400, 2000 or 4000 mg/kg bw per day for 104 weeks (cited in EFSA 2007 as PCR 1014). Overall, body weight, body weight gain and feed consumption of both sexes were lower compared at 400, 2000 and 4000 mg/kg bw treated animals compared to the control group. Body weights of treated mice were 93%–95% and 90%–94% of controls, at week 52 and week 78, respectively. Feed consumption in both sexes was also lower at 400, 2000 and 4000 mg/kg bw, and the decrease was most apparent from week 15 to 77. No carcinogenic effect of neotame was reported up to the highest dose tested (4000 mg/kg bw). The incidence of hepatocellular adenomas in males and of bronchiolar-alveolar carcinomas in females at 4000 mg/kg bw were higher (but did not reach the statistical significance) when compared to controls. The increased incidences of both tumours were considered not to be treatment-related due to the absence of pre-neoplastic changes.

In another carcinogenicity study, Sprague–Dawley CD rats were administered neotame at dietary concentrations of 50, 500 or 1000 mg/kg bw per day (cited in EFSA 2007 as PCR 1000). The exposure to neotame-containing diet started in utero (85 animals/sex/group), by treating the male parent F0 generation for 4 weeks before and throughout mating, and the female F0 generation throughout gestation, lactation to weaning at day 21 after littering. The F1 generation (75 animals/sex/group) was exposed to the same dose levels as the parental generation for 104 weeks. No carcinogenic effect of neotame up to the highest doses tested (1000 mg/kg bw) was reported. The incidence of neoplasms in all treatment groups of both sexes was not statistically significantly different compared to controls, except for an increased incidence of adenomas in kidneys of males at 50 mg/kg bw. This finding was not considered as treatment-related, since no tumours were observed in the high-dose group.

The AFC Panel considered that the decreases in body weight or body weight gain (in all treated groups of rats and in mice receiving doses of 400 mg neotame/kg bw per day or higher) were linked to reduced palatability of the neotame-containing diet and not to toxicity of the compound, therefore these were not considered appropriate endpoints for setting NOAELs for neotame.

Overall, the FAF Panel agreed with the conclusions of the AFC Panel that there were no adverse effects in chronic and carcinogenicity studies.

3.5.6 | Reproductive and developmental toxicity

No new data were received following the call for biological and toxicological data,[6] and no new data were identified from the literature search. In the evaluation of neotame (E 961) by EFSA, the AFC Panel reviewed the available reproductive and embryofetal toxicity studies in rats and an embryofetal study in rabbits (EFSA, 2007). These unpublished studies were part of the original submission of an application on neotame as a new food additive and they are reported in Appendix B of the previous EFSA 2007 opinion. A summary of these studies is presented in this section. More details can be found in the 2007 EFSA opinion.

In a two-generation reproductive study in Sprague–Dawley rats, neotame was administered in diet at 0, 100, 300 or 1000 mg/kg bw per day to F0 males for 10 weeks and females for 4 weeks before mating ($n = 28$/sex/dose) (cited in EFSA 2007 as PCR 1001); treatment continued from mating to gestation, then during the lactation period until weaning of F1 offspring at PND21. Selected F1 offspring were dosed as their mothers from about PND28 for 10 weeks before mating; F2

offspring were terminated at PND21. In F0, body weight and feed intake were reduced in males at 100 and 1000 mg/kg bw per day throughout premating, and body weight gain was reduced in females at 1000 mg/kg bw per day during premating and gestation (95% of control mean on gestation day (GD) 20). In F1, pup weight was reduced at 300 and 1000 mg/kg bw per day in males on PND1 (approximately 10% and 13% respectively) and in both sexes on PND21 (approximately 9–10% and 13%, respectively). Female F1 body weights remained reduced at 1000 mg/kg bw per day throughout gestation (approximately 9%). The reduced body weights were accompanied by reduced feed consumption (92% compared to control). In F2 offspring, body weight was reduced on PND21 in both sexes at 1000 mg/kg bw per day. There were no treatment-related effects on F0 and F1 reproduction parameters (oestrus cycle, mating performance, fertility, gestation length, parturition and gestation index, litter size, sex ratio and offspring viability indices), or on F1 and F2 physical development, auditory and visual reflex performance, activity and learning ability, and physical maturation, or on adult F0 and F1 organ macropathology or organ weights. In summary, neotame administered in the diet to rats for two generations reduced feed intake and body weight gain at 1000 mg/kg bw per day in F0, F1 and F2, and also at 300 mg/kg in F1, but had no effect on reproduction parameters at up to 1000 mg/kg bw per day (cited in EFSA, 2007 as PCR 1001).

In a dietary female fertility and embryo/fetal development study, female Sprague–Dawley CD rats (24/group) were administered neotame in the diet at 0, 100, 300 or 1000 mg/kg bw per day for 4 weeks before mating with untreated males, and throughout gestation until necropsy on GD20 (cited in EFSA 2007 as PCR 0999). There was an immediate but transient decrease in feed intake on day 1 of premating period at 300 and 1000 mg/kg bw per day, resulting in decreased body weight gain (57% of controls) during the first week of premating at 1000 mg/kg bw per day. Body weight gain at 1000 mg/kg bw per day increased to 87% of controls by the end of the 4-week premating period, and to 98% of controls on GD20. Treatment had no effects on clinical signs, pregnancy rate, corpora lutea count, pre- and post-implantation loss, litter size, fetal survival, fetal weights or incidences of skeletal and soft tissue abnormalities. In summary, oral neotame at up to 1000 mg/kg bw per day had no adverse effects on female fertility or embryofetal development.

In an embryo/fetal development study, pregnant New Zealand White rabbits ($n = 29$/dose) were dosed neotame 0, 50, 150 or 500 mg/kg bw per day by gavage on GD 6–19 (cited in EFSA, 2007 as PCR 1023). Additional satellite animals were used to measure plasma concentration of neotame and its metabolite NC-00751 on days 1, 8 and 14 of treatment. One 150 mg/kg bw per day dosed dam died due to a gavage error. Total litter loss occurred in one control and one 500 mg/kg bw per day dosed dam. At 500 mg/kg bw per day, one dam died on day 20 and two aborted on GD 28 or 29; these three rabbits likely aborted as a result of reduced feed consumption and body weight losses. One 150 mg/kg bw per day dam had four small and grossly abnormal fetuses; this dam also exhibited reduced feed consumption and body weight. The AFC Panel concluded that since the malformations were not dose-related and because rabbits are particularly sensitive to body weight loss, the findings are not toxicologically relevant. In dams with litters carried to term, feed consumption and body weight gain were not significantly affected by treatment, and there were no adverse embryofetal effects. The AFC Panel concluded that oral neotame at up to 500 mg/kg did not affect embryofetal development in the rabbit (derived from EFSA 2007, citing). The Panel agreed with this conclusion.

The AFC Panel noted that there were no adverse effects on reproduction or embryofetal development in these studies except those associated with possible maternal toxicity in the rabbit study. In these studies, body weights and feed intake were decreased from 100, 300 or 500 mg/kg bw per day depending on the type of the study (EFSA, 2007).

Overall, the FAF Panel agreed with the conclusions of the AFC Panel that there were no adverse effects on reproduction or embryo/fetal development.

3.5.7 | Other studies

Two studies retrieved from the literature search, which do not directly contribute to the safety assessment of neotame as a food additive, are summarised below.

In one study, three experiments were conducted to evaluate the effects of neotame on diet preference, performance and haematological and biochemical parameters of weaned piglets (Zhu et al., 2016). The first experiment was a diet preference study to determine if pigs consumed more diet containing neotame than diet without; 48 weaned piglets were randomly assigned to eight pens (six pigs/pen). Each pen was equipped with two feeders, one containing a maize-soybean meal-based diet, the other the same diet supplemented with 30 mg/kg neotame. The experiment consisted of 5 days adaption period and a 10-day test period (15 days in total). The amount of feed consumed was determined daily. The results showed that the diet containing 30 mg/kg neotame was consumed significantly more ($p < 0.05$) than the control diet during day 3, 6, 7, 9, 10 and the entire experimental period of 15 days (feed intake of 218 g/day in the control diet vs. 248 g/day in the diet containing 30 mg/kg neotame; the average diet preference percentage was 46.7% in the control diet vs. 53.3% in the diet containing 30 mg neotame /kg diet).

The second experiment assessed the effect of neotame on the performance of weaned piglets. A total of 216 weaned piglets were allocated to six different diets (six pens/group; six pigs/pen): basal diet supplemented with 0, 10, 20, 30, 40 or 50 mg neotame /kg diet. The experiment included two phases (I: day 1–22 and II: day 23–35). The results showed that the average daily feed intake (ADFI) significantly ($p < 0.05$) increased with increasing dietary neotame concentrations during phase I and the entire experimental period, whereas the average daily body weight gain (ADG) and average daily feed intake (ADFI) significantly increased ($p < 0.05$) with increasing neotame dietary levels, during phase I and II (35 days in total). No statistically significant effects ($p > 0.05$) were observed on feed conversion ratio (FCR) during any period.

The third experiment was conducted to assess the effects of neotame on haematological and biochemical parameters of weaned piglets. The RoB evaluation for these endpoints was Tier 3 (Appendix B) and not further considered.

The Panel noted that in weaned piglets, increased intake of feed added up to 30 mg neotame/kg diet was associated with increased body weight gain (of approximately 10%, $p < 0.05$) as compared to controls kept on the basal feed with no neotame added.

In another study (Yin et al., 2020), the effects of different sweeteners (including neotame) on taste preference behaviour were assessed in mice in a two-bottle preference experiment. Totally 150 male mice were divided into 10 groups (one for each sweetener), according to their body weight and 5 mice were housed in a cage. All the mice were adapted for 5 days with free access to drinking water using double bottle method before the experiment. During the experiment one bottle contained water and the other contained a neotame solution (0.001, 0.01, 0.1, 1, 10 mM or equivalent to 0.0004; 0.0038; 0.0378; 0.3745; 3.7847 mg/L). The consumption of the solutions in the two bottles was recorded every 48 h. The mice showed a tendency to avoid neotame at concentrations of 1 mM or above.

3.5.8 | Human studies

No new data were received following the call for biological and toxicological data.[6]

In the evaluation of neotame (E 961) by EFSA, the AFC Panel reviewed different human studies available at the time (cited in EFSA, 2007 as PCR 1035, PCR 1111, PCR 1112, PCR 1145, PCR 1113, PCR 1114 and PCR 1115). These unpublished studies were part of the original submission of an application on neotame as a new food additive. They are reported in Appendix B of the previous EFSA 2007 opinion. A summary of these studies is presented below. More details can be found in the 2007 EFSA opinion.

The studies from a clinical testing programme evaluated the metabolism and pharmacokinetics, and the safety of neotame in healthy volunteers (number of subjects varying from 12 to 150) and those with non-insulin dependent diabetes mellitus (NIDDM) (18 males and 19 females). Based on the results of those studies, the AFC Panel concluded that neotame was well tolerated by healthy and diabetic (NIDDM) human subjects at dose levels up to 1.5 mg/kg bw per day. In two of these studies, the most common adverse effect was headache, and its incidence was similar among treated and non-treated (12 /sex/group) of 21%, 29% and 15% in the placebo, 0.5 mg/kg bw and 1.5 mg/kg bw per day dose groups, respectively, or of 31%, 29% and 27% of healthy subjects (76 males and 75 females in total) in the placebo, 0.5 mg/kg bw and 1.5 mg/kg bw per day dose groups, respectively. The number of subjects reporting adverse events was not statistically significantly different between the treatment groups. The AFC Panel considered the adverse effects observed in these studies not treatment-related (cited as PCR1113 and PCR 1114 in EFSA, 2007).

One new study was retrieved from the literature (Gibbons et al., 2024). This study was a randomised crossover trial conducted in 53 overweight and obese volunteers (males and females aged 18–60 years) recruited across two centres in UK and France. The aim of the study was to assess the acute (1 day) and repeated (2-weeks) effects of three formulations of biscuits containing either Stevia, neotame or sucrose on appetite (desire to eat + hunger +fullness + intake) and endocrine responses (glucose, insulin, ghrelin and glucagon-like peptide). The study was assessed and evaluated as moderate risk of bias (Tier 2) due to lack of information on the amount of neotame used for sweetening the biscuits (although it can be assumed that the amount used was made to match the sweetness of sucrose in the biscuit, which was ~25 g/100 g). Compared to sucrose the neotame-containing biscuits did not affect glucose, ghrelin, glucagon-like peptide 1 and pancreatic polypeptide. However, postprandial insulin (2-h iAUC) was lower after neotame intake ($d = -0.71$, $p < 0.001$) compared to sucrose. No differences were found between acute versus two-week of daily consumption. Gastrointestinal symptoms including excess gas/wind and bloating were more frequently reported in the neotame compared to sucrose group (24 vs. 49) but no formal statistical comparison was performed by the authors. Given the subjective nature of these symptoms, a replication in a different study would be needed to draw any firm conclusions.

3.6 | Environmental considerations

The applicable EU legislation on food additives establishes that the approval of food additives should consider, among other factors, also the protection of the environment. In the framework of the re-evaluation of neotame under Regulation (EU) No 257/2010, EFSA has not received any information from IBOs or any other interested party in relation to any environment risks of neotame (E 961) however it became aware of data and information available in the public domain.

An extensive review collating published data on neotame (E 961) was performed to identify evidence of potential adverse effects on the environment (Agriculture and Environment Research Unit, University of Hertfordshire (AERU), 2021) resulting from the use of neotame (E 961) as a food additive. In addition to this review, the Panel considered additional papers retrieved in the updated literature search in the present assessment (see Appendix B).

As reported in Section 3.5.1, the Panel considered that neotame is rapidly absorbed and pre-systemically metabolised in humans. Any systemic intact neotame is likely excreted in the urine along with its metabolites. NC-00751 has been identified as the primary metabolite of neotame (about 80% of the dose excreted in all species tested). Therefore, neotame and NC-00751 has the potential to reach the aquatic environment via wastewater. Neotame may also reach the terrestrial environment (e.g. via agricultural fertilisation with sewage sludge or flood events). In AERU (2021) a LogKow ranging between 2.4 and 3.8 is reported, generally a LogKow higher than 3 would indicate a potential for bioconcentration.

In the review from AERU (2021), any data regarding the environmental fate, persistence or ecotoxicology of neotame, or for its primary metabolite NC-00751 were not identified. In the updated literature search some additional papers, with respect to the one already identified in AERU (2021), on the occurrence in the environment of neotame were retrieved. Very limited data on techniques for removing neotame from the water, including laboratory simulation studies, were retrieved (Ma et al., 2021; Shen et al., 2023; Yue et al., 2024; Yue, Guo, et al., 2023). In addition, two publications from the European Commission Horizon 2020 'Sweets project' reporting on the life cycle assessments of the production of non-nutritive sweeteners were retrieved (Suckling et al., 2023, 2025).

Regarding the occurrence of neotame in the environment, according to Nofre and Tinti (2000), in water the major degradation pathway of neotame is the hydrolysis of the methyl ester group into NC-00751 and methanol, this process being relatively slow. Taking into consideration this, together with the information on the ADME of neotame, it is expected that neotame metabolites (and to a lesser extent neotame as parent compound) would be present in the environment.

No studies were identified in which NC-00751 was analysed in environmental samples, both in AERU (2021) and in the updated literature search. Some papers providing analytical measurements of neotame in environmental matrices were retrieved.

Neotame was analysed in wastewater and reported as not detected in Kokotou & Thomaidis, 2013, Lakade et al., 2018 and Scheurer et al., 2009, Alves et al., 2021, Yue, Li, et al., 2023. In Gvozdić et al. (2020), the measured concentrations of neotame in wastewater ranged from not detected to 129 ng/L. Comparable concentrations were reported in Gan et al. (2013), Linhoff (2022), Guo et al. (2021) and Shen et al., 2023.

Limited information was available on the concentrations of neotame in surface water. In some papers (Alves et al., 2021; Berset & Ochsenbein, 2012; Lakade et al., 2018; Scheurer et al., 2009), neotame was reported as not detected in samples of surface water from different countries (Switzerland, Spain, Germany, Brasil). In Gvozdić et al. (2020, 2023), the concentrations of neotame in samples of surface water from Serbia ranged from not detected to 27 ng/L. Gan, Sun, Feng, et al. (2013); Gan, Sun, Wang, and Feng (2013) and Yue, Guo, et al. (2023) reported concentrations of neotame in surface water and/or costal water collected in China within the same range of concentrations. Gvozdić et al. (2020, 2023) reported neotame and aspartame as the only sweeteners detected in river sediment in their studies (2–56 ng/g).

The international platform of chemical monitoring (IPCHEM)[21] includes data on the concentration of neotame in different environmental compartments from several EU and non-EU countries (from EMPODAT[22]). According to this database, neotame was below the reported range of LODs in biota (e.g. fish, otters: range 0.00125–5 ng/g wet weight), wastewater (range 1–2 ng/L), surface water (range 0.63–1.25 ng/L), sediment (5 ng/g dry weight) and groundwater (range 1–5 ng/L).

Overall, neotame was not consistently detected in environmental samples. Based on the available information, when detected in surface water or river sediment, its concentration was in the range of ng/L or ng/g. The available data on the environmental concentrations of neotame are based on isolated monitoring studies and are not part of systematic monitoring programmes. The available studies included data from both EU and non-EU countries and may not be fully representative of the European situation. These data therefore give only a rough indication of the concentration of neotame in the environment. Ecotoxicological studies were not available, and no environmental data were available on the primary metabolite and degradation product of neotame, NC-00751.

3.7 | Discussion

The present opinion deals with the re-evaluation of neotame (E 961), authorised as food additive in the European Union (EU) in accordance with Annex II and Annex III to Regulation (EC) No 1333/2008 on food additives.

Neotame was evaluated previously by JECFA (JECFA, 2004) and by the former Panel of EFSA on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (EFSA, 2007). Both committees established an ADI of 2 mg/kg bw per day based on the application of a 100-fold safety factor to the NOAEL of 200 mg/kg bw from a 52-week dog study.

The Panel noted that the majority of the data provided by one IBO for neotame (E 961) during the calls for data, were the same to the ones submitted at the time of the relevant EFSA assessment in 2007 (EFSA, 2007).

Neotame is the chemically manufactured compound N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester obtained by reaction under hydrogen pressure of N-L-α-aspartyl-L-phenylalanine 1-methyl ester (aspartame) with 3,3-dimethylbutyraldehyde in methanol in presence of a palladium/carbon catalyst, as specified in Commission Regulation (EU) No 231/2012.

Considering the manufacturing process, the food additive is a product of chemical synthesis using well defined chemical precursors, involving several separation and purification steps. The analytical data provided for the purity of neotame (E 961) were in all analysed samples higher than 98%. This being the case, the Panel recommends raising the minimum percentage of neotame from 97% to at least 98% in the assay requirement outlined in the EU specifications.

The main impurity of neotame (E 961) is also a degradation product (de-esterified form) called N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751). The Panel noted that in the EU specifications, this impurity is referred as

[21] https://ipchem.jrc.ec.europa.eu, accessed on 21.3.2025.

[22] EMPODAT compiles data from monitoring, survey, research/technical studies provided by NORMAN member organisations (https://www.normandata.eu/?q=Home). The reason of data collection therefore may vary by dataset and/or data provider.

N-[(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine and the name should be changed to N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine, to be consistent with the chemical name of the food additive itself. The Panel also noted that the major degradation product NC-00751 is also the primary metabolite which implies that NC-00751 is tested in the toxicological studies. Therefore, the safety of this impurity is covered by the toxicity data available on neotame and does not give rise to concern as an impurity at the level of up to 1.5%.

The Panel noted that apart from NC-00751, the parameter 'other related substances' (Not more than 2.0%) as described in the specifications of JECFA (2003), is not included in the current EU specifications (Table 1). Such structurally related impurities of E 961 are included in Appendix A of the EFSA, 2007 and in Appendix D, Table D.1 of the current opinion. The potential exposure to the 'other related substances', resulting from the use of E 961 was individually assessed using the Threshold of Toxicological Concern (TTC) approach (Appendix E). The 'other related substances' (Appendix D, with the exception of L-phenylalanine and 3,3-dimethylbutylamine (Cramer Class I)) were classified into Cramer Class III (Appendix F, Table F.2). In order not to exceed the respective TTC value (1.5 μg/kg bw per day), their presence individually should be less than 0.9% in the food additive. Considering the exposure estimates, the purity value of neotame and the analytical data provided for the 'other related substances', the Panel noted that no concern is identified for those impurities and thus, there is no need for them to be included in the EU specifications.

In addition, the Panel considered that, even though neotame (E 961) is optically active, no information on its specific optical rotation is included in the EU specifications. As a result, the Panel considered that information on its specific optical rotation should be included in the EU specifications.

Considering the microbiological data submitted by the IBO, the Panel considered that a microbiological contamination is unlikely and, therefore, it is not necessary to recommend inclusion of microbiological criteria in the EU specifications for E 961.

Regarding toxic elements, the Panel assessed the risk that would result if (i) Pb was present in E 961 at the current maximum limit in the EU specifications and if (ii) Pd was present in E 961 at the rounded up highest measured value provided by one IBO. Considering the results of the exposure to the toxic element Pb, the Panel noted that its presence in E 961 at the current specification limit value would not give rise to concern. For Pd, the estimate of exposure coming from the uses and use levels of E 961 is only a very small fraction of the respective PDE, an indication of no health concern. This being the case, the Panel did not consider it necessary to propose a specification limit for Pd in the EU specifications for E 961.

Taking into account the data provided additionally for As, Cd and Hg and taking also into account that the manufacturing process is an organic synthesis with several separation and purification steps and so systematic contamination by these inorganics is not anticipated, the Panel did not see a need to recommend additional specifications for As, Cd and Hg.

The Panel noted that the current EU specifications include a solubility value of *'4.75% (w/w) at 60°C in water, and solubility in ethanol and ethyl acetate*'. According to the analytical data provided for the solubility of neotame (E 961), the Panel considered that neotame is 'sparingly soluble in water, very soluble in ethanol', in line with the JECFA specifications. Therefore, the Panel suggests an amendment to the description of the solubility of E 961 in line with the JECFA specifications. In addition, the Panel took note of the consideration made by one IBO that 'ethyl acetate is not a particularly useful solvent for food applications, nor for analytical purposes' and concurred with the suggestion to remove the provision on solubility in ethyl acetate from the EU specifications of E 961.

Based on the submitted data, the Panel noted that the presence of small particles including nanoparticles in the food additive E 961 has been reported by the IBO under the examined conditions.

The reported solubility value (e.g. 12.6 g/L at 25°C) for neotame (E 961), is lower than the threshold value of 33.3 g/L, as a decision criterion for demonstrating that the material does not require specific assessment at the nanoscale (EFSA Scientific Committee, 2021b). The Panel noted that the maximum use levels of neotame (E 961) reported by the food industry for various food categories (see Annex A1) do not exceed 60 mg/kg and the highest MPL does not exceed 250 mg/kg (FC 5.3). For tabletop sweeteners the food additive is allowed *quantum satis*, however these are not intended to be consumed as such and will be largely diluted in beverages and, accordingly, particles would be expected to dissolve. Taking into account the maximum reported use levels, the MPLs, the reported solubility value and the volume of gastric secretion (ranging from 215 mL within a single meal to 2000 mL daily; ICRP, 2002; Mudie et al., 2014), the Panel considered that full dissolution of E 961, is to be expected in foods and/or in the gastrointestinal tract and that ingested particles (if any) would not persist. Considering this, the Panel concluded that there is no concern with regard to the potential presence of small particles, including nanoparticles, in neotame (E 961) at the reported uses and use levels and considered that the food additive can be assessed following the conventional risk assessment, i.e. EFSA Guidance for submission for food additive evaluations (EFSA ANS Panel, 2012).

The Panel noted that, based on the submitted information along with considerations of the structure and characteristics of neotame, E 961 is stable in most food categories, but is prone to hydrolysis and other degradation pathways, particularly at very low pH and high temperature, to form mainly NC-00751, along with NC-00777, NC-00779 and NC00764, plus other minor degradation products (Appendix D).

The potential hazard related to nitrosation of neotame and its degradation products in the gastrointestinal tract was considered in the previous EFSA opinion (2007). Based on the lack of formation of nitrosated by-products in simulated gastric juice, and on the lack of mutagenicity in vitro of nitrosated neotame and its metabolite NC-00751 (de-esterified neotame), the AFC Panel concluded that the hypothetical nitrosation of neotame, should it occur, was of no concern with respect to genotoxicity (EFSA, 2007). The FAF Panel agrees with this conclusion.

No new data were received following the call for biological and toxicological data.[6] From the literature search performed,[23] the new studies which were considered as relevant, according to the inclusion criteria reported in the protocol on hazard identification and characterisation (EFSA, 2020a; EFSA FAF Panel, 2023), were one study in pigs to evaluate the efficacy of neotame added to feed (Zhu et al., 2016), one study on taste preference behaviour in mice (Yin et al., 2020) and one study on appetite in humans (Gibbons et al., 2024).

In the safety evaluation of neotame (E 961) as a basis for its authorisation as a food additive, the AFC Panel reviewed several toxicological studies in animal and human studies available at the time (EFSA, 2007). In this evaluation, a summary of these studies is presented. In accordance with the revised protocol (EFSA FAF Panel, 2023), studies previously evaluated by the SCF or EFSA and considered for setting an ADI were subjected to a RoB evaluation. In the case of neotame, the study on which the current ADI was based (i.e. the 52-week dog study, cited in EFSA 2007 as PCR 1017) was considered of moderate RoB (allocated as Tier 2). According to the criteria outlined in the revised protocol (EFSA FAF Panel, 2023), being the key studies from the previous evaluation rated as tier 1 or tier 2 in the RoB assessment, the Panel considered it appropriate not to re-assess the other previously evaluated studies.

The FAF Panel considered that neotame is rapidly absorbed and pre-systemically metabolised in both animals and humans. Any systemic intact neotame is likely to be excreted in the urine along with its metabolites. A significant fraction of neotame metabolites may result from pre-systemic metabolism. The data are consistent with biliary excretion in dogs. The Panel noted that the major degradation product N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751) is also the primary metabolite of neotame, which implies that the available toxicological dataset also informs on the toxicological profile of NC-00751, being an impurity, a degradation product and a metabolite. The Panel noted that NC00751 is formed by de-esterification resulting in a molecule which is structurally similar to the parent compound along with methanol (Figure 4).

The results of in vitro genotoxicity assays indicate that neotame does not induce gene mutation. A possible clastogenic or aneugenic activity was suggested by the results from an in vitro micronucleus assay, in which neotame induced the formation of micronuclei in the presence and absence of metabolic activation. Considering the available experimental data which include a negative in vitro chromosomal aberration assay, the Panel considered that the positive findings in the in vitro MN assay are due to aneugenicity. Negative results were obtained in an in vivo *bone marrow* micronucleus test in mice. Considering the evidence of bioavailability of oral administered neotame, as demonstrated by toxicokinetic data, the Panel concluded that genotoxic effects due to systemic exposure to neotame and its metabolites are not expected. However, the available data were insufficient to address the possible aneugenic effects of neotame at the site of contact (oesophagus and stomach).

Therefore, in the absence of validated test methods to assess aneugenicity in the upper GIT, following the relevant EFSA Guidance (EFSA Scientific Committee, 2021a), the Panel compared the concentrations resulting in induction of micronuclei in vitro with the MPL for beverages (FC 14.1.3 and FC 14.1.4), i.e. assuming the highest concentration of neotame in oesophagus and stomach is equal to the MPL. To identify a reference point for aneugenicity assessment, the in vitro data from the micronucleus test were modelled and a benchmark dose (BMD) analysis using the EFSA 'Bayesian BMD' webtool was conducted. The FAF Panel decided to use historical negative control data reported by the IBO (Documentation provided to EFSA No. X) for deriving the respective BMRs for the 4 h (without metabolic activation) and 24 h MN assays, as explained in Section 3.5.2 and Appendix G. Considering that the data used for this analysis were concentrations and not doses, the Panel considered it appropriate to use the term benchmark concentration (BMC), rather than BMD.

As a worst-case scenario, the Panel compared the 24 h BMCL to the concentration equal to the currently authorised maximum permitted levels (MPL) of 20 mg/L in FC 14.1.3 and FC 14.1.4 corresponding to beverages (see Table 4; Annex Exposure, table A.5). The Panel noted that this BMCL (156 mg/L) is approximately eight-fold higher than the MPL of 20 mg/L for neotame (E 961) in beverages. Comparing the reported use levels and analytical data in beverages (7 mg/L for FC 14.1.3 and 2.8 mg/L for FC 14.1.4, respectively) with the BMCL would result in a difference of more than an order of magnitude.

Therefore, the Panel considered that aneugenic effects at the site of contact are not expected to occur under the intended condition of use of neotame. Furthermore, as recommended by the EFSA SC statement (EFSA Scientific Committee, 2017), other toxicological data may assist in supporting this consideration; thus, the available additional data were considered. There was no indication for carcinogenicity in two studies in mice and rats with doses up to 1000 mg/kg bw per day and no adverse effects in the GIT (see Section 3.5.4) or other systemic adverse effects in additional studies, including three reproductive and developmental toxicity studies (see Section 3.5.5).

Overall, the Panel considered that there is no concern for genotoxicity of neotame (E 961) at the maximum permitted levels or reported use levels.

Concerning systemic toxicity, in the previous evaluation an isolated elevation of serum hepatic alkaline phosphatase (AP) activity in two separate studies in dogs (i.e. a 13-week- and a 52-week study on which the current ADI is based on), as well as in a 13-week rat study (but not in a separate 52-week rat study) was considered as an indicator of adverse liver effects. However, in these studies an increase in serum AP activity was not accompanied by an elevation in other serum biomarkers of liver injury, and no histopathological changes indicative of toxicity to the liver were reported.

The FAF Panel therefore considered that the increase in hepatic AP activity in the serum in the dog studies was not a marker of an adverse effect. The Panel noted that this conclusion is in line with ongoing discussion in the scientific literature on the relevance of AP as an indicator of hepatotoxicity in dogs, in the absence of additional hepatotoxic findings, e.g. Yokoyama et al. 2019 (https://pubmed.ncbi.nlm.nih.gov/31568816/).

[23]To cover the period from the latest EFSA evaluation with an overlap of 1 year (i.e. 2006) until 2 October 2024.

A review of the other endpoints in the available toxicological database did not indicate an adverse effect for neotame at the highest doses tested in rodents, i.e. up to doses of 3000 mg/kg bw per day in a 13-week study in rats, up to 1000 mg/kg bw per day in a 52-week chronic and 104-week carcinogenicity studies in rats, and up to 4000 mg/kg bw per day in mice dosed for 104 weeks. The Panel noted that at the highest doses in all studies there was reduced feed intake associated with decreased body weight gain. The Panel agreed with the AFC Panel (EFSA, 2007) that these decreases in body weight or body weight gain should not be considered as indicative of toxicity but related to reduced palatability in rodents (Flamm et al., 2003; Mayhew et al., 2003).

In light of the above, a reference point (RP) of 1000 mg/kg bw per day from a 52-week chronic and 104-week carcinogenicity studies in rats is chosen to derive the ADI for neotame. However, the Panel noted that methanol, along with NC-00751, is a primary metabolite of neotame. Furthermore, formaldehyde, which is derived from methanol metabolism, is genotoxic (NTP, 2014).

The Panel therefore considered that, in order to establish a health-based guidance value (HBGV) for neotame, the resulting exposure to methanol and its metabolite formaldehyde from the use of neotame should be taken into account to ensure that the dietary exposure to the food additive does not raise a concern.

Based on the RP of 1000 mg/kg bw per day, a five-fold higher ADI of 10 mg/kg bw per day would be derived. This would increase the steady state concentration of methanol and the peak level of formaldehyde. In humans and primates, toxicity of methanol is mediated through its metabolites and not the parent molecule. Therefore, the exposure to formaldehyde was estimated using worst-case assumptions (see Appendix C). The resulting steady state level and peak level of formaldehyde in humans were compared to data in rats on the concentration of formaldehyde in blood and the interindividual variation (Kleinnijenhuis et al., 2013). In the rat study the coefficient of variation was 30% for the formaldehyde concentration. An increase of the ADI to 10 mg/kg bw per day would result in an increase of about 4% (steady state level) and 18.5% (peak level) of formaldehyde background, which is within the observed background variation of 30%.

With respect to phenylalanine, in agreement with the previous EFSA opinion (EFSA, 2007), the FAF Panel considered that this amino acid is only a minor metabolite of neotame (less than 1% of the dose). The FAF Panel considered that the amount of phenylalanine which could be potentially formed from neotame (E 961) at the proposed ADI of 10 mg/kg bw per day would be safe for the general population, including individuals heterozygous for the phenylalanine hydroxylase gene, as it would increase the physiological phenylalanine concentration by less than 1% (EFSA ANS Panel, 2013).

Based on these considerations, the Panel considered appropriate to establish an ADI for neotame (E 961) of 10 mg/kg bw per day based on application of a default 100-fold uncertainty factor to the NOAEL of 1000 mg/kg bw per day from a 52-week chronic and 104-week carcinogenicity studies in rats.

Dietary exposure to neotame (E 961) was estimated according to different exposure scenarios based on consumers only as described in Section 3.4. Currently, neotame (E 961) is an authorised food additive in the EU in 34 food categories, while concentration data were available for only five categories as described in Section 3.3.1.

In general, neotame (E961) is not commonly used in Europe, as indicated by the Mintel GNPD and literature, therefore, the Panel considered the exposure to neotame (E 961) from its use as food additive, to be an overestimation in all scenarios.

The Panel considered *the refined brand-loyal exposure assessment scenario*, the most appropriate exposure scenario for the risk assessment. In this *scenario*, mean exposure to neotame (E 961) ranged from less than or equal to 0.01 mg/kg bw per day in all age groups to 0.05 mg/kg bw per day in infants and toddlers. The 95th percentile of exposure ranged from 0.01 mg/kg bw per day in adolescents, adults and the elderly to 0.16 mg/kg bw per day in toddlers.

The Panel noted that exposure estimates at the mean and P95 in all population groups for all scenarios did not exceed the ADI of 10 mg/kg bw per day for neotame (E 961).

4 | UNCERTAINTY

The uncertainties, and the direction of the uncertainty, related to the exposure assessments are summarised in Table 7 of Section 3.4.2. Overall, the Panel considered the dietary exposure estimates of neotame (E 961) for all exposure scenarios to be overestimates of the current dietary exposure to the food additive.

In the genotoxicity assessment of neotame, the only positive findings were reported in in vitro MN assays suggesting a possible aneugenic effect at the site of contact. To identify a suitable reference point for aneugenicity assessment, the Panel performed a BMD analysis of the in vitro MN data. Detailed uncertainties around the BMC (i.e. BMCU/BMCL), the selection of the BMR and the duration of the exposure are described in Section 3.5.2 and Appendix G.

Overall, the uncertainties addressed above were considered not to affect the conclusions on the safety of neotame (E 961).

5 | CONCLUSIONS

The Panel established an ADI of 10 mg/kg bw per day for neotame based on the NOAEL of 1000 mg/kg bw per day, the highest dose tested and by applying an uncertainty factor of 100. Accordingly, this ADI replaces the ADI of 2 mg/kg bw per day established by the EFSA AFC Panel (2007).

The dietary exposure estimates of neotame (E 961) for the different population groups of all exposure scenarios did not exceed the ADI.

The Panel concluded that there is no safety concern for neotame (E 961) at the currently permitted and reported uses and use levels.

6 | RECOMMENDATIONS

The Panel recommends the EC to consider:

- changing the name of the impurity N-[(3,3-dimethylbutyl)-L-α-aspartyl]-L- phenylalanine to N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine in the EU specifications;
- raising the minimum percentage of neotame from 97% to at least 98% in the assay requirement outlined in the EU specifications;
- introducing information on the specific optical rotation in the EU specifications of E 961; and
- revising the solubility parameter to 'sparingly soluble in water, very soluble in ethanol'.

DOCUMENTATION AS PROVIDED TO EFSA

1. International Sweeteners Association, 2019. Submission of data in response to the call for technical and toxicological data on sweeteners authorised as food additives in the EU. Submitted on 02 December 2019.
2. International Sweeteners Association, 2020. Submission of data in response to the for technical data on sweeteners authorised as food additives in the EU. Submitted on 20 February 2020.
3. International Sweeteners Association, 2020. Clarification on the data submitted in response to the call for technical and toxicological data on sweeteners authorised as food additives in the EU. Submitted on 20 December 2020.
4. International Sweeteners Association, 2021. Neotame: micronucleus test in human lymphocytes in vitro. Covance Laboratories Ltd., Study Number 8453412, issued 22 February 2021.
5. International Sweeteners Association, 2021. Clarification on the data submitted in response to the call for technical and toxicological data on sweeteners authorised as food additives in the EU. Submitted on 16 March 2021.
6. International Sweeteners Association, 2021. Clarification on the data submitted in response to the call for technical and toxicological data sweeteners authorised as food additives in the EU. Submitted on 23 March 2021.
7. International Sweeteners Association, 2022. Submission of data in response to the call for data on genotoxicity data on sweeteners. Submitted on 05 October 2020.
8. International Sweeteners Association, 2023. Clarification on the data submitted in response to the call for technical and toxicological data sweeteners authorised as food additives in the EU. Submitted on 19 September 2023.
9. International Sweeteners Association, 2023. Clarification on the data submitted in response to the call for technical and toxicological data sweeteners authorised as food additives in the EU. Submitted on 25 September 2023.
10. Manus Bio, 2024. Submission of data in response to the call for data on genotoxicity data on sweeteners. Submitted on 12 August 2024.
11. International Sweeteners Association, 2023. Clarification on the data submitted in response to the call for technical and toxicological data on sweeteners authorised as food additives in the EU. Submitted on 22 May 2023.
12. Association of Italian Sweets and Pasta Industries. Submission of data on use levels of neotame (E 961) in response to call for food additives usage level and/or concentration data in food and beverages intended for human consumption. Data submitted on 28 September 2018.
13. European Fruit Juice Association. Submission of data on use levels of neotame (E 961) in response to call for food additives usage level and/or concentration data in food and beverages intended for human consumption. Data submitted on 23 November 2018.

ABBREVIATIONS

ADG	average daily body weight gain
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, excretion
AFC Panel	Panel on Food Additives Flavourings, Processing Aids and Materials in Contact With Food
AgNPsFP	filter paper-based silver nanoparticle
ALP	aluminium phosphide
ANS Panel	Panel on Food Additives and Nutrient Sources Added to Food
AP	alkaline phosphatase
As	arsenic
BMDL	benchmark dose (lower confidence limit)
bw	bodyweight
CAD	charged aerosol detection

CAS	Chemical Abstracts Service
Cd	cadmium
CONTAM Panel	Panel on Contaminants in the Food Chain
CTA	chemical and technical assessment
DAD	diode array detection
ELSD	evaporative light scattering detection
FAF Panel	Panel on Food Additives and Flavourings
FCR	feed conversion ratio
GNPD	Mintel'S Global New Products Database
Hg	mercury
HPLC	high-performance liquid chromatography
HPLC–UV	high-performance liquid chromatography with ultraviolet detection
IBO	interested business operator
ICP–MS	inductively coupled plasma-mass spectrometry
IQ	intelligence quotient
IUPAC	International Union Of Pure And Applied Chemistry
JECFA	Joint Fao/Who Expert Committee On Food Additives
LD	laser diffraction
LOEL	lowest observed effect leve
LOQ	limit of quantification
MALDI–TOF MS	matrix assisted laser desorption/ionisation time-of-flight mass spectrometry
MOE	margin of exposure
MOS	margin of safety
MPL	maximum permitted level
MS	mass spectroscopy
MS/MS	tandem mass spectrometry
NOAEL	no observed adverse effect level
NTA	nanoparticle tracking analysis
Pb	lead
Pd	palladium
PDE	permitted daily exposure
QS	*quantum satis*
QTRAP MS	quadrupole-trap mass spectrometry
RH	relative humidity
RP	Reference Points
RP-HPLC	reversed-phase high-performance liquid chromatography
SERS	surface-enhanced raman scattering
TEM	transmission electron microscopy
TTC	threshold of toxicological concern
USP	United States Pharmacopeia
UV	ultraviolet
w/w	weight per weight

ACKNOWLEDGEMENTS

The Panel wishes to thank the following for the support provided to this scientific output: Antonios Douros, Trine Husøy, Evangelia Ntzani, Antonio Rivas Cornejo, Romina Shah, Heather Wallace and Maged Younes. The Panel wishes to acknowledge all European competent institutions, Member State bodies and other organisations that provided data for this scientific output.

REQUESTOR

European Commission

QUESTION NUMBER

EFSA-Q-2011-00740

PANEL MEMBERS

Monica Andreassen, Gabriele Aquilina, Maria Bastos, Polly Boon, Laurence Castle, Biagio Fallico, Reginald FitzGerald, Maria Jose Frutos Fernandez, Bettina Grasl-Kraupp, Ursula Gundert-Remy, Rainer Gürtler, Eric Houdeau, Marcin Kurek, Maria Henriqueta Louro, Patricia Morales, and Sabina Passamonti.

MAP DISCLAIMER

The designations employed and the presentation of material on any maps included in this scientific output do not imply the expression of any opinion whatsoever on the part of the European Food Safety Authority concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

REFERENCES

Agriculture and Environment Research Unit, University of Hertfordshire, Lewis, K. A., & Tzilivakis, J. (2021). Review and synthesis of data on the potential environmental impact of artificial sweeteners. *EFSA Supporting Publications*, *18*, 127. https://doi.org/10.2903/sp.efsa.2021.EN-6918

Alves, P. D. C. C., Rodrigues-Silva, C., Ribeiro, A. R., & Rath, S. (2021). Removal of low-calorie sweeteners at five Brazilian wastewater treatment plants and their occurrence in surface water. *Journal of Environmental Management*, *289*, 112561. https://doi.org/10.1016/j.jenvman.2021.112561

Bathinapatla, A., Kanchi, S., Singh, P., Sabela, M. I., & Bisetty, K. (2014). Determination of neotame by high-performance capillary electrophoresis using ß-cyclodextrin as a chiral selector. *Analytical Letters*, *47*(17), 2795–2812. https://doi.org/10.1080/00032719.2014.924008

Benigni, R., Bossa, C., Jeliazkova, N., Netzeva, T., & Worth, A. (2008). The Benigni/Bossa rulebase for mutagenicity and carcinogenicity-a module of Toxtree. *Mutation Research*, *659*, 248–261.

Berset, J.-D., & Ochsenbein, N. (2012). Stability considerations of aspartame in the direct analysis of artificial sweeteners in water samples using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS). *Chemosphere*, *88*(5), 563–569. https://doi.org/10.1016/j.chemosphere.2012.03.030

Cheng, S., Wang, S., Zheng, M., Jin, Y., Li, J., Zhang, M., Li, X. L., & Min, J. Z. (2024). Simultaneous analysis of natural and artificial sweeteners in sugar-free drinks and urine samples by column-switching UHPLC-charged aerosol detection method. *Journal of Chromatography A*, *1713*, 464533. https://doi.org/10.1016/j.chroma.2023.464533

Committee to Review the Formaldehyde Assessment in the National Toxicology Program 12th Report on Carcinogens, Board on Environmental Studies and Toxicology, Division on Earth and Life Sciences, & National Research Council. (2014). *Review of the formaldehyde assessment in the National Toxicology Program 12th report on carcinogens*. National Academies Press (US). https://doi.org/10.17226/18948

Cramer, G. M., Ford, R. A., & Hall, R. L. (1978). Estimation of toxic hazard—A decision tree approach. *Food and Cosmetics Toxicology*, *16*, 255–276. https://doi.org/10.1016/S0015-6264(76)80522-6

de Sousa, R. C. S., Gomides, M. D., Costa, K., Cunha, M. R. R., Almeida, M. D., Custódio, F. B., & Gloria, M. B. A. (2024). Optimization and validation of an analytical method for the determination of sweeteners in beverages by HPLC-ELSD. *Food Analytical Methods*, *17*(2), 207–225. https://doi.org/10.1007/s12161-023-02562-w

Detry, P., Willame, P., Van Hoeck, E., Van Loco, J., & Goscinny, S. (2022). Development, validation and application of multi-class methods for the analysis of food additives by liquid chromatography coupled to tandem mass spectrometry. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment*, *39*(8), 1349–1364. https://doi.org/10.1080/19440049.2022.2085887

Dewinter, L., Casteels, K., Corthouts, K., Van de Kerckhove, K., Van der Vaerent, K., Vanmeerbeeck, K., & Matthys, C. (2016). Dietary intake of non-nutritive sweeteners in type 1 diabetes mellitus children. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, *33*(1), 19–26. https://doi.org/10.1080/19440049.2015.1112039

EFSA (European Food Safety Authority). (2007). Neotame as a sweetener and flavour enhancer - scientific opinion of the panel on food additives, Flavourings, processing aids and materials in contact with food. *EFSA Journal*, *5*(11), 581. https://doi.org/10.2903/j.efsa.2007.581

EFSA (European Food Safety Authority). (2011). Use of the EFSA comprehensive European food consumption database in exposure assessment. *EFSA Journal*, *9*(3), 2097. https://doi.org/10.2903/j.efsa.2011.2097

EFSA (European Food Safety Authority). (2015). Scientific report on principles and process for dealing with data and evidence in scientific assessments. *EFSA Journal*, *13*(5), 4121. https://doi.org/10.2903/j.efsa.2015.4121

EFSA (European Food Safety Authority). (2020a). Outcome of the public consultation on a draft protocol for the assessment of hazard identification and characterisation of sweeteners. *EFSA Supporting Publications*, *17*(2), EN-1803. https://doi.org/10.2903/sp.efsa.2020.EN-1803

EFSA (European Food Safety Authority). (2020b). Outcome of the public consultation on a draft protocol for assessing exposure to sweeteners as part of their safety assessment under the food additives re-evaluation programme. *EFSA Supporting Publications*, EN-1913. https://doi.org/10.2903/sp.efsa.2020.EN-1913

EFSA AFC Panel (EFSA Panel on Food Additives Flavourings, Processing Aids and Materials in Contact With Food). (2007). Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on the presence of 1,2-Benzisothiazolin-3-one as an impurity in saccharin use as a food additive. *EFSA Journal*, *5*(1), 416. https://doi.org/10.2903/j.efsa.2007.416

EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources added to Food). (2013). Scientific Opinion on the re-evaluation of aspartame (E 951) as a food additive. *EFSA Journal*, *11*(12), 3496. https://doi.org/10.2903/j.efsa.2013.3496

EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources). (2012). Guidance for submission for food additive evaluations. *EFSA Journal*, *10*(7), 2760. https://doi.org/10.2903/j.efsa.2012.2760

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain). (2010). Scientific opinion on Lead in food. *EFSA Journal*, *8*(4), 1570. https://doi.org/10.2903/j.efsa.2010.1570

EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings). (2023). Revised protocol on Hazard identification and characterisation of sweeteners. *EFSA Journal*. https://doi.org/10.5281/ZENODO.7788969

EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings). (2024). Revised protocol for assessing exposure to sweeteners as part of their safety assessment under the food additives re-evaluation programme. *EFSA Journal*. https://zenodo.org/records/13912094

EFSA Scientific Committee. (2009). Guidance of the Scientific Committee on transparency in the scientific aspects of risk assessment carried out by EFSA. Part 2: General principles. *EFSA Journal*, *7*(4), 1051. https://doi.org/10.2903/j.efsa.2009.1051

EFSA Scientific Committee. (2011). Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. *EFSA Journal*, *9*(9), 2379. https://doi.org/10.2903/j.efsa.2011.2379

EFSA Scientific Committee. (2012). Guidance on selected default values to be used by the EFSA scientific committee, scientific panels and units in the absence of actual measured data. *EFSA Journal*, *10*(3), 2579. https://doi.org/10.2903/j.efsa.2012.2760

EFSA Scientific Committee. (2017). Scientific Opinion on the clarification of some aspects related to genotoxicity assessment. *EFSA Journal*, *15*(12), 5113. https://doi.org/10.2903/j.efsa.2017.5113

EFSA Scientific Committee. (2019). Guidance on the use of the threshold of toxicological concern approach in food safety assessment. *EFSA Journal*, *17*(6), 5708. https://doi.org/10.2903/j.efsa.2019.5708

EFSA Scientific Committee. (2021a). Scientific Opinion on the guidance on aneugenicity assessment. *EFSA Journal*, *19*(8), 6770. https://doi.org/10.2903/j.efsa.2021.6770

EFSA Scientific Committee. (2021b). Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles. *EFSA Journal*, *19*(8), 6769. https://doi.org/10.2903/j.efsa.2021.6769

EFSA Scientific Committee. (2022). Guidance on the use of the benchmark dose approach in risk assessment. *EFSA Journal*, *20*(10), 7584. https://doi.org/10.2903/j.efsa.2022.7584

Flamm, W. G., Blackburn, G. L., Comer, C. P., Mayhew, D. A., & Stargel, W. W. (2003). Long-term food consumption and body weight changes in neotame safety studies are consistent with theallometric relationship observed for other sweeteners and during dietary restrictions. *Regulatory Toxicology and Pharmacology*, *38*, 144–156. https://doi.org/10.1016/S0273-2300(03)00075-8

Gan, Z., Sun, H., Feng, B., Wang, R., & Zhang, Y. (2013). Occurrence of seven artificial sweeteners in the aquatic environment and precipitation of Tianjin, China. *Water Research*, *47*(14), 4928–4937. https://doi.org/10.1016/j.chroma.2012.11.081

Gan, Z., Sun, H., Wang, R., & Feng, B. (2013). A novel solid-phase extraction for the concentration of sweeteners in water and analysis by ion-pair liquid chromatography–triple quadrupole mass spectrometry. *Journal of Chromatography A*, *1274*, 87–96. https://doi.org/10.1016/j.chroma.2012.11.081

Gibbons, C., Beaulieu, K., Almiron-Roig, E., Santiago Navas-Carretero, J., Martínez, A., O'Hara, B., O'Connor, D., Nazare, J.-A., Le Bail, A., Rannou, C., Hardman, C., Wilton, M., Kjølbæk, L., Scott, C., Moshoyiannis, H., Raben, A., Harrold, J. A., Halford, J. C. G., & Finlayson, G. (2024). Acute and two-week effects of neotame, stevia rebaudioside M and sucrose-sweetened biscuits on postprandial appetite and endocrine response in adults with overweight/obesity-a randomised crossover trial from the SWEET consortium. *eBioMedicine*, *28*(102), 105005. https://doi.org/10.1016/j.ebiom.2024.105005

Guo, W., Li, J., Liu, Q., Shi, J., & Gao, Y. (2021). Tracking the fate of artificial sweeteners within the coastal waters of Shenzhen city, China: From wastewater treatment plants to sea. *Journal of Hazardous Materials*, *414*, 125498. https://doi.org/10.1016/j.jhazmat.2021.125498

Gvozdić, E., Bujagić, I. M., Đurkić, T., & Grujić, S. (2023). Untreated wastewater impact and environmental risk assessment of artificial sweeteners in river water and sediments of the Danube River basin in Serbia. *Environmental Science and Pollution Research*, *30*(35), 84583–84594. https://doi.org/10.1007/s11356-023-28348-5

Gvozdić, E., Matić Bujagić, I., Đurkić, T., & Grujić, S. (2020). Trace analysis of artificial sweeteners in environmental waters, wastewater and river sediments by liquid chromatography–tandem mass spectrometry. *Microchemical Journal*, *157*, 105071. https://doi.org/10.1016/j.microc.2020.105071

Han, M., Wei, W., Lu, H., & Zhang, Z. (2020). Rapid and sensitive detection of neotame in instant grain beverages by paper-based silver nanoparticles substrates. *Micro & Nano Letters*, *15*(15), 1099–1104. https://doi.org/10.1049/mnl.2020.0298

Henschel, J., Schriewer, A., Hayen, H., & Jiang, W. (2016). Analysis of artificial sweeteners by HILIC-MS method. *LC GC Europe*, *29*(7), 401–401.

Hu, F., Xu, L., Luan, F., Liu, H., & Gao, Y. (2013). Determination of neotame in non-alcoholic beverage by capillary zone electrophoresis. *Journal of the Science of Food and Agriculture*, *93*(13), 3334–3338. https://doi.org/10.1002/jsfa.6181

Hu, J. A., Mao, W., Chen, W. T., Zhang, S. Y., Liu, Z., He, Y. X., Fu, C. H., Wang, C. X., Wu, J., & Zou, P. (2023). A QTRAP-based mass spectrometry method for the detection and confirmation of nine sweeteners in Chinese rice wine. *Journal of Food Measurement and Characterization*, *17*(6), 6298–6306. https://doi.org/10.1007/s11694-023-02108-y

ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use). (2019). Guideline for elemental impurities Q3D (R1). https://www.ema.europa.eu/en/documents/scientific-guideline/international-conference-harmonisation-technical-requirements-registration-pharmaceuticals-human-use_en-32.pdf

ICRP (International Commission on Radiological Protection). (2002). Basic anatomical and physiological data for use in radiological protection reference values. *Annals of the ICRP*, *32*(3–4), 277. https://doi.org/10.1016/S0146-6453(03)00002-2

Iwakoshi, K., Tahara, S., Uematsu, Y., Yamajima, Y., Miyakawa, H., Monma, K., Kobayashi, C., & Takano, I. (2019). Development of a highly sensitive liquid chromatography with tandem mass spectrometry method for the qualitative and quantitative analysis of high-intensity sweeteners in processed foods. *Journal of Chromatography A*, *1592*, 64–70. https://doi.org/10.1016/j.chroma.2019.01.036

Janvier, S., Goscinny, S., Le Donne, C., & Van Loco, J. (2015). Low-calorie sweeteners in food and food supplements on the Italian market. *Food Additives & Contaminants. Part B, Surveillance*, *8*(4), 298–308. https://doi.org/10.1080/19393210.2015.1094829

JECFA (Joint FAO/WHO Expert Committee on Food Additives). (2000). Safety evaluation of certain food additives and contaminants: Fifty-third report of the joint FAO/WHO expert committee on food additives. *WHO Technical Report Series*, *896*, 128. https://iris.who.int/bitstream/handle/10665/42378/WHO_TRS_896.pdf?sequence=1&isAllowed=y

JECFA (Joint FAO/WHO Expert Committee on Food Additives). (2003). Specifications for neotame. FNP 52, Add. 11. FAO, Rome.

JECFA (Joint FAO/WHO Expert Committee on Food Additives). (2004). Combined compendium of food additives specifications. Analytical methods, test procedures and laboratory solutions used by and referenced in the food additive specifications. Geneva, Switzerland. https://www.fao.org/3/a0691e/a0691e00.htmJEC

Kleinnijenhuis, A. J., Staal, Y. C., Duistermaat, E., Engel, R., & Woutersen, R. A. (2013). The determination of exogenous formaldehyde in blood of rats during and after inhalation exposure. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *52*, 105–112. https://doi.org/10.1016/j.fct.2012.11.008

Kokotou, M. G., & Thomaidis, N. S. (2013). Determination of eight artificial sweeteners in wastewater by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *Analytical Methods*, *5*(16), 3825–3833. https://doi.org/10.1039/C3AY40599K

Kokotou, M. G., & Thomaidis, N. S. (2018). Characterization of the retention of artificial sweeteners by hydrophilic interaction liquid chromatography. *Analytical Letters*, *51*(1–2), 49–72. https://doi.org/10.1080/00032719.2017.1326124

Krmela, A., Kharoshka, A., Schulzova, V., Pulkrabova, J., & Hajslova, J. (2020). A simple LC-MS multi-Analyte method to determine food additives and caffeine in beverages. *LC GC Europe*, *33*(7), 327–335.

Kumari, A., Arora, S., Choudhary, S., Singh, A. K., & Kaushik, R. (2023). Comparative study of aspartame and neotame stability in ice cream and cake. *Indian Journal of Dairy Science*, *76*(1), 10–18. https://doi.org/10.33785/IJDS.2023.v76i01.002

Kumari, A., Arora, S., Choudhary, S., Singh, A. K., & Tomar, S. K. (2017). Comparative stability of aspartame and neotame in yoghurt. *International Journal of Dairy Technology*, *71*(1), 81–88. https://doi.org/10.1111/1471-0307.12381

Kumari, A., Arora, S., Singh, A. K., & Choudhary, S. (2016). Development of an analytical method for estimation of neotame in cake and ice cream. *LWT-Food Science and Technology*, *70*, 142–147. https://doi.org/10.1016/j.lwt.2016.02.045

Kumari, A., Choudhary, S., Arora, S., & Sharma, V. (2016). Stability of aspartame and neotame in pasteurized and in-bottle sterilized flavoured milk. *Food Chemistry*, *196*, 533–538. https://doi.org/10.1016/j.foodchem.2015.09.082

Kuo, B., Beal, M. A., Wills, J. W., White, P. A., Marchetti, F., Nong, A., Barton-Maclaren, T. S., Houck, K., & Yauk, C. L. (2022). Comprehensive interpretation of in vitro micronucleus test results for 292 chemicals: From hazard identification to risk assessment application. *Archives of Toxicology*, *96*, 2067–2085. https://doi.org/10.1007/s00204-022-03286-2

Lakade, S. S., Zhou, Q., Li, A., Borrull, F., Fontanals, N., & Marcé, R. M. (2018). Hypercrosslinked particles for the extraction of sweeteners using dispersive solid-phase extraction from environmental samples. *Journal of Separation Science*, *41*(7), 1618–1624. https://doi.org/10.1002/jssc.201701113

Le Donne, C., Mistura, L., Goscinny, S., Janvier, S., Cuypers, K., D'Addezio, L., Sette, S., Catasta, G., Ferrari, M., Piccinelli, R., Van Loco, J., & Turrini, A. (2017). Assessment of dietary intake of 10 intense sweeteners by the Italian population. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *102*, 186–197. https://doi.org/10.1016/j.fct.2017.02.014

Lephalala, M., Kanchi, S., Sabela, M. I., & Bisetty, K. (2020). Electrochemical enzymatic biosensing of Neotame supported by computational methods. *Electroanalysis*, *32*(12), 2669–2680. https://doi.org/10.1002/elan.202060208

Li, L. P., Fan, S., Liu, W., Zhang, N., Wang, L. L., Zhao, R., & Wu, G. H. (2020). Simultaneous determination of 9 kinds of sweeteners in liquor by ultra performance liquid chromatography-tandem mass spectrometry. *Journal of Food Safety and Quality*, *11*(7), 2216–2222.

Linhoff, B. (2022). Deciphering natural and anthropogenic nitrate and recharge sources in arid region groundwater. *Science of the Total Environment*, *848*, 157345. https://doi.org/10.1016/j.scitotenv.2022.157345

Lorenzo, R. A., Pena, M. T., Fernández, P., González, P., & Carro, A. M. (2015). Artificial sweeteners in beverages by ultra performance liquid chromatography with photodiode array and liquid chromatography tandem mass spectrometry. *Food Control*, *47*, 43–52. https://doi.org/10.1016/j.foodcont.2014.06.035

Ma, X., Liu, Z., Yang, Y., Zhu, L., Deng, J., Lu, S., Li, X., & Dietrich, A. M. (2021). Aqueous degradation of artificial sweeteners saccharin and neotame by metal organic framework material. *The Science of the Total Environment*, *761*, 143181. https://doi.org/10.1016/j.scitotenv.2020.143181

Mayhew, D. A., Comer, C. P., & Stargel, W. W. (2003). Food consumption and body weight changes with neotame, a new sweetener with intense taste: Differentiating effects of palatability from toxicity in dietary safety studies. *Regulatory Toxicology and Pharmacology*, *38*(2), 124–143. https://doi.org/10.1016/S0273-2300(03)00074-6

Moher, D., Liberati, A., Tetzlaff, J., & Altman, D. G. (2009). Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. *PLoS Medicine*, *6*(7), e1000097. https://doi.org/10.1371/journal.pmed.1000097

Mudie, D. M., Murray, K., Hoad, C. L., Pritchard, S. E., Garnett, M. C., Gordon, L., Amidon, G. L., Gowland, P. A., Spiller, R. C., Amidon, G. E., & Marciani, L. (2014). Quantification of gastrointestinal liquid volumes and distribution following a 240 mL dose of water in the fasted state. *Molecular Pharmaceutics*, *11*, 3039–3047. https://doi.org/10.1021/mp500210c

Nicoluci, I. G., da Silva, B. S., Braga, P. A. C., & Bragotto, A. P. A. (2023). Simultaneous determination of nine high-intensity sweeteners in liquid and powder tabletop sweeteners. *Food Additives and Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, *40*(10), 1298–1306. https://doi.org/10.1080/19440049.2023.2238836

Nofre, C., & Tinti, J.-M. (2000). Neotame: Discovery, properties, utility. *Food Chemistry*, *69*(3), 245–257. https://doi.org/10.1016/S0308-8146(99)00254-X

NTP-OHAT (National Toxicology Program- Office of Health Assessment and Translation). (2019). Handbook for conducting a literature-based health assess-ment using OHAT approach for systematic review and evidence integration. 101. https://ntp.niehs.nih.gov/sites/default/files/ntp/ohat/pubs/handbookmarch2019_508.pdf.

OECD (Organization for Economic Co-operation and Development). (2014-2016). *Test No. 487: In vitro mammalian cell micronucleus test, OECD guidelines for the testing of chemicals*. OECD Publishing.

OECD. (2016a). Test no. 473: In vitro mammalian chromosomal aberration test. In *OECD guidelines for the testing of chemicals, section 4*. OECD Publishing. https://doi.org/10.1787/9789264264649-en

OECD. (2016b). Test no. 490: In vitro mammalian cell gene mutation tests using the thymidine kinase gene. In *OECD guidelines for the testing of chemicals, section 4*. OECD Publishing. https://doi.org/10.1787/9789264264908-en

Ordoñez, E. Y., Rodil, R., Quintana, J. B., & Cela, R. (2015). Determination of artificial sweeteners in beverages with green mobile phases and high temperature liquid chromatography-tandem mass spectrometry. *Food Chemistry*, *169*, 162–168. https://doi.org/10.1016/j.foodchem.2014.07.132

Powley, M. W., Sobol, Z., Johnson, G. E., Clark, R. W., Dalby, S. M., Ykoruk, B. A., Galijatovic-Idrizbegovic, A., Mowery, M. D., & Escobar, P. A. (2024). N-nitrosamine impurity risk assessment in pharmaceuticals: Utilizing in vivo mutation relative potency comparison to establish an acceptable intake for NTTP. *Regulatory Toxicology and Pharmacology*, *152*, 105681. https://doi.org/10.1016/j.yrtph.2024.105681

Prakash, I., Corliss, G., Ponakala, R., & Ishikawa, G. (2002). Neotame: The next-generation sweetener. *Food Technology*, *56*(7), 36–45.

SCF (Scientific Committee on Food). (2001). Guidance on submissions for food additive evaluations by the Scientific Committee on Food. 42 pp. https://ec.europa.eu/food/fs/sc/scf/out98_en.pdf.

Scheurer, M., Brauch, H. J., & Lange, F. T. (2009). Analysis and occurrence of seven artificial sweeteners in German waste water and surface water and in soil aquifer treatment (SAT). *Analytical and Bioanalytical Chemistry*, *394*(6), 1585–1594. https://doi.org/10.1016/j.scitotenv.2014.02.047

Sezgin, B., Arli, G., & Can, N. Ö. (2021). Simultaneous HPLC-DAD determination of seven intense sweeteners in foodstuffs and pharmaceuticals using a core-shell particle column. *Journal of Food Composition and Analysis*, *97*, 103768. https://doi.org/10.1016/j.jfca.2020.103768

Shah, R., De Jager, L. S., & Begley, T. H. (2014). Development and single-laboratory validation of an improved method for the determination of cyclamate in foods using liquid chromatography/tandem mass spectrometry. *Journal of AOAC International*, *97*(6), 1651–1655. https://doi.org/10.5740/jaoacint.14-042

Shen, G., Lei, S., Li, H., Yu, Q., Wu, G., Shi, Y., Xu, K., Ren, H., & Geng, J. (2023). Occurrence and removal of four artificial sweeteners in wastewater treatment plants of China. *Environmental Science: Processes & Impacts*, *25*(1), 75–84. https://doi.org/10.1039/D2EM00351A

Soeteman-Hdernandez, L. G., Fellows, M. D., Johnson, G. E., & Slob, W. (2015). Correlation of *in vivo* versus *in vitro* benchmark doses (bmds) derived from micronucleus test data: a proof of concept study. *Toxicological Sciences*, *148*, 355–367.

Soyseven, M., Sezgin, B., & Arli, G. (2023). The development and validation of a novel, green, sustainable and eco-friendly HPLC-ELSD method approach for the simultaneous determination of seven artificial sweeteners in various food products: An assessment of the greenness profile of the developed method with an analytical eco-scale, NEMI, GAPI and AGREE. *Microchemical Journal*, *193*, 109225. https://doi.org/10.1016/j.microc.2023.109225

Suckling, J., Morse, S., Murphy, R., Raats, M., Astley, S., Ciruelos, A., Crespo, A., Halford, J. C. G., Harrold, J. A., Le-Bail, A., Koukouna, E., Musinovic, H., Raben, A., Roe, M., Scholten, J., Scott, C., & Westbroek, C. (2025). Environmental life cycle assessment of drink and yoghurt products using non-nutritive sweeteners and sweetness enhancers in place of added sugar: the SWEET project. *The International Journal of Life Cycle Assessment*, *30*(2), 251–272. https://doi.org/10.1007/s11367-024-02375-x

Suckling, J., Morse, S., Murphy, R., Raats, M., Astley, S., Halford, J. C. G., Harrold, J. A., Le-Bail, A., Koukouna, E., Musinovic, H., Raben, A., Roe, M., Scholten, J., Scott, C., & Westbroek, C. (2023). Environmental life cycle assessment of production of the non-nutritive sweeteners aspartame (E951) and neotame (E961) from chemical processes: The SWEET project. *Journal of Cleaner Production*, *424*, 138854. https://doi.org/10.1016/j.jclepro.2023.138854

Wang, M., Ling, L., Wang, S., & Ding, C. F. (2024). A homogeneous binary matrix assisted laser desorption/ionization time-of-flight mass spectrometry assay for determination of artificial sweeteners in beverages. *Food Chemistry*, *460*(Pt 2), 140597. https://doi.org/10.1016/j.foodchem.2024.140597

Yin, K. J., Xie, D. Y., Zhao, L., Fan, G., Ren, J. N., Zhang, L. L., & Pan, S. Y. (2020). Effects of different sweeteners on behavior and neurotransmitters release in mice. *Journal of Food Science and Technology*, *57*, 113–121. https://doi.org/10.1007/s13197-019-04036-6

Yokoyama, Y., Ono, A., Yoshida, M., Matsumoto, K., & Saito, M. (2019). Toxicological significance of increased serum alkaline phosphatase activity in dog studies of pesticides: Analysis of toxicological data evaluated in Japan. *Regulatory Toxicology and Pharmacology*, *109*, 104482.

Yue, J., Guo, W., Li, D., Zhu, Y., Zhao, Q., Wang, A., & Li, J. (2023). Seasonal occurrence, removal and mass loads of artificial sweeteners in the largest water reclamation plant in China. *Science of the Total Environment*, *856*, 159133. https://doi.org/10.1016/j.scitotenv.2022.159133

Yue, J., Guo, W., Zhu, Y., Li, D., Liang, S., Cao, R., Wang, A., & Li, J. (2024). Insights on the degradation mechanism of neotame using UV/periodate: Roles of reactive species, kinetics, and pathways. *Chemical Engineering Journal*, *495*, 153059. https://doi.org/10.1016/j.cej.2024.153059

Yue, Y., Li, L., Qu, B., Liu, Y., Wang, X., Wang, H., & Chen, S. (2023). Levels, consumption, and variations of eight artificial sweeteners in the wastewater treatment plants of Dalian city, China. *Science of the Total Environment*, *892*, 163867. https://doi.org/10.1016/j.scitotenv.2023.163867

Zeller, A., Duran-Pacheco, G., & Guérard, M. (2017). An appraisal of critical effect sizes for the benchmark dose approach to assess dose–response relationships in genetic toxicology. *Archives of Toxicology*, *91*, 3799–3807. https://doi.org/10.1007/s00204-017-2037-3

Zhang, J., Grandi, E., Fu, H., Saravanan, T., Bothof, L., Tepper, P. G., Thunnissen, A. W. H., & Poelarends, G. J. (2020). Engineered C-N Lyase: Enantioselective synthesis of chiral synthons for artificial dipeptide sweeteners. *Angewandte Chemie (International Ed. in English)*, *59*(1), 429–435. https://doi.org/10.1002/anie.201910704

Zhu, K., Chen, Y., Yu, L., Hou, C., Qiao, X., & Wang, T. (2023). Determination of neotame in various foods by high-performance liquid chromatography coupled with ultraviolet and mass spectrometric detection. *Food Chemistry*, *416*, 135863. https://doi.org/10.1016/j.foodchem.2023.135863

Zhu, L., Wang, G., Dong, B., Peng, C., Tian, Y., & Gong, L. M. (2016). Effects of sweetener neotame on diet preference, performance and hematological and biochemical parameters of weaned piglets. *Animal Feed Science and Technology*, *214*, 86–94. https://doi.org/10.1016/j.anifeedsci.2016.02.013

Zygler, A., Wasik, A., & Namieśnik, J. (2010). Retention behaviour of some high-intensity sweeteners on different SPE sorbents. *Talanta*, *82*(5), 1742–1748. https://doi.org/10.1016/j.talanta.2010.07.070

Zygler, A., Wasik, A., Kot-Wasik, A., & Namieśnik, J. (2012). Content of high-intensity sweeteners in different categories of foods available on the polish market. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, *29*(9), 1391–1401. https://doi.org/10.1080/19440049.2012.699475

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), Castle, L., Andreassen, M., Aquilina, G., Bastos, M. L., Boon, P., Fallico, B., FitzGerald, R., Frutos Fernandez, M. J., Grasl-Kraupp, B., Gundert-Remy, U., Gürtler, R., Houdeau, E., Kurek, M., Louro, H., Morales, P., Passamonti, S., Batke, M., Bruzell, E., … Lodi, F. (2025). Re-evaluation of neotame (E 961) as food additive. *EFSA Journal*, *23*(7), e9480. https://doi.org/10.2903/j.efsa.2025.9480

APPENDIX A

Exposure calculations to the toxic elements from the use of neotame (E 961)

One IBO provided analytical data on the levels of lead (Pb), mercury (Hg), cadmium (Cd), arsenic (As) and palladium (Pd) in different analysed samples (Documentation provided to EFSA n. 1, 3, 7, 8). Specifically, the results of analyses in five samples were reported as < 3 mg/kg for As and as < 1 mg/kg for Pb, using the USP 231 limit test (Documentation provided to EFSA n. 1, 3). The Panel noted that the results were reporting limits and that the method USP 231 is a colorimetric test that is rather insensitive and non-specific, and its use has been generally discontinued. Furthermore, the IBO provided additional results of analyses in five other samples of the food additive for Pb, which was reported as < 1 mg/kg in all samples. The Panel noted that no analytical protocol was provided for these analyses and that the results were reporting limits. One sample of E 961 was also analysed by inductively coupled plasma–mass spectrometry (ICP–MS), according to USP 233. In the single sample, Pb was reported as below the reporting limit of 0.10 mg/kg, As as below the reporting limit of 0.10 mg/kg, Cd as below the reporting limit of 0.02 mg/kg, and Hg as below the reporting limit of 0.01 mg/kg (Documentation provided to EFSA n. 3). After EFSA's request to the IBO to provide analytical data on palladium (Pd), meaning the catalyst used, the IBO provided data on five samples of neotame analysed by ICP-MS in the same laboratory, with Pd ranging from below the reporting limit of 0.10 to 0.41 mg/kg, Pb being below the reporting limit of 0.10 mg/kg in all samples, As being below the reporting limit of 0.10 mg/kg in all samples, Cd being below the reporting limit of 0.02 mg/kg in all samples and Hg being below the reporting limit of 0.01 mg/kg in all samples (Documentation provided to EFSA n. 7, 8).

The Panel noted that no information on the lowest technologically achievable levels for the toxic elements in E 961 was provided by the IBO, although requested by EFSA.

The potential exposure to lead (limit included in the EU specifications) and palladium (catalyst used in the manufacturing of E 961) from the use of neotame (E 961) was calculated by assuming that the impurities are present in the food additive up to a limit value, and then by calculation pro-rata to the estimates of exposure to the food additive itself.

With regard to the dietary exposure to E 961, the Panel considered the refined brand-loyal exposure assessment scenario (Table 6). For the current assessment, the highest exposure levels for the mean and 95th percentile among the different population groups were considered, i.e. 0.05 and 0.16 mg/kg bw per day, for toddlers, respectively.

For Pb and Pd, the potential levels of these toxic elements in E 961 combined with the estimated exposure levels to E 961, (presented in Table A.1) result in exposure estimates that can be compared with the following reference points (RP).

TABLE A.1 Reference points for toxic elements potentially present in the food additive E 961.

Element/RP	Basis/reference
Lead (Pb)/0.5 mg/kg bw per day ($BMDL_{01}$)	The reference point is based on a study demonstrating perturbation of intellectual development in children with the critical response size of 1 point reduction in IQ. The EFSA CONTAM Panel mentioned that a 1 point reduction in IQ is related to a 4.5% increase in the risk of failure to graduate from high school and that a 1 point reduction in IQ in children can be associated with a decrease of later productivity of about 2%. A risk cannot be excluded if the exposure exceeds the $BMDL_{01}$ (MOE lower than 1). EFSA CONTAM Panel (2010)
Palladium (Pd)/2 µg/kg bw per day (PDE)	The PDE for oral exposure is based on a LOEL of 1.2 mg/kg bw per day in a lifetime study with mice taking into account five modifying factors and a human body weight of 50 kg. ICH (2019)

Abbreviations: bw, body weight; $BMDL_{01}$, benchmark dose (lower confidence limit); LOEL, lowest observed effect level; MOE, margin of exposure; PDE, permitted daily exposure; RP, reference point.

The risk assessment of toxic elements helps to determine whether there could be a possible health concern if these impurities and constituents would be present at the limit values in the food additive. The assessment is performed by calculating the MOE (margin of exposure) by dividing the reference point (e.g. BMDL) by the exposure estimate (Table 6).

Concerning the possible presence of toxic elements, the existing specifications for E 961 have a limit value only for lead of 'not more than 1 mg/kg' (Table 1). The IBO provided analytical data on the levels of lead, arsenic, mercury, cadmium and palladium, in commercial samples of neotame (E 961) (Section 3.1.1).

The Panel assessed the risk that would result if (i) Pb was present in E 961 at the current maximum limit in the EU specification and if (ii) Pd was present in E 961 at the rounded up highest measured value provided by one IBO.

The outcome of the risk assessment of the Panel for these scenarios is presented in Table A.2.

Taking into account the data provided additionally for As, Cd and Hg and taking also into account that the manufacturing process is an organic synthesis with several separation and purification steps and so systematic contamination by these inorganics is not anticipated, the Panel did not see a need to recommend additional specifications for As, Cd and Hg.

The Panel emphasised that the choice of the maximum limit values as well as other considerations, such as on multiple sources of exposure to conclude on the maximum limits for toxic elements in the specifications is in the remit of risk management. The numbers used here are merely taken to support the risk assessment of these toxic element as presented below.

TABLE A.2 Risk assessment for toxic elements from the use of E 961.

	(i) Considering the presence of Pb at the current limit of the EU specifications for E 961 (Commission Regulation (EU) No 231/2012)
Exposure to E 961 (mg/kg bw per day)	**MOE for Pb at 1.0 mg/kg**
0.05[a]	10,000
0.16[b]	3125
	(ii) Considering the presence of Pd at the rounded up highest measured value provided by one IBO (Documentation provided to EFSA n. 3)
Exposure to E 961 (mg/kg bw per day)	**% of the PDE for Pd at 0.5 mg/kg**
0.05[a]	0.001
0.16[b]	0.004

[a]Highest exposure level among the different population groups (refined brand-loyal scenario – toddlers – mean) (Table 6).

[b]Highest exposure level among the different population groups (refined brand-loyal scenario scenario – toddlers – 95th percentile) (Table 6).

Considering the results of the exposure to the toxic element Pb, the Panel noted that its presence in E 961 at the current specification limit value would not give rise to concern. For Pd, the estimate of exposure coming from the uses and use levels of E 961 is only a very small fraction of the respective PDE, an indication of no health concern. This being the case, the Panel did not consider it necessary to propose a specification limit for Pd in the EU specifications for E 961.

APPENDIX B

Tailored protocol for the assessment of hazard identification and characterisation applied for neotame (E 961)

No new data were received following the call for biological and toxicological data.[6] From the literature search performed,[24] the new studies which were considered as relevant, according to the inclusion criteria reported in the protocol on hazard identification and characterisation (EFSA, 2020a; EFSA FAF Panel, 2023), were one study in pigs on efficacy of neotame addition to animal feed (Zhu et al., 2016) and one study on taste preference behaviour (Yin et al., 2020). These studies were summarised but did not undergo the RoB and WoE process, according to the protocol. However, a third experiment from the Zhu et al. (2016) study which assessed the effects of neotame on haematological and biochemical parameters of weaned piglets underwent a RoB evaluation for these endpoints and was evaluated as Tier 3 and therefore not further considered. One study in human (Gibbons et al., 2024) underwent a RoB evaluation and considered to be Tier 2.

In the safety evaluation of neotame (E 961) by EFSA (2007) as a basis for its authorisation, the AFC Panel had reviewed several toxicological studies in animal and human studies available at that time. In this opinion, a summary of these studies is presented and more details can be found in the 2007 EFSA opinion. In accordance with the revised protocol (EFSA FAF Panel, 2023), studies previously evaluated by the SCF or EFSA and considered for setting an ADI were also subjected to a RoB evaluation. In the case of neotame, the study on which the ADI was based (i.e. the 52-week dog study, cited in EFSA, 2007 as PCR 1017) was considered of moderate risk of bias (RoB) (allocated as Tier 2). Therefore, according to the criteria outlined in the revised protocol (EFSA FAF Panel, 2023), the Panel considered it appropriate not to re-assess the other previously considered studies.

Please refer to steps **1.1 to 1.10** of the general protocol for hazard identification and characterisation of sweeteners (EFSA, 2020a; EFSA FAF Panel, 2023).

For **step 1.11** (Extensive literature searches), the general principles reported in the protocol applied. The extensive literature searches were conducted in the three selected databases with the search strings and criteria applied as follow:

Web of Science (SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-EXPANDED, IC.)

Date of the search: 1/10/2024

Set	Query
#1	TS = (Neotame OR E961 OR "E 961" OR "165450–17-9")
#2	TS = (Neotame OR E961 OR "E 961" OR "165450–17-9") AND PY = 2006–2100
#3	TS = (Neotame OR E961 OR "E 961" OR "165450–17-9") AND PY = 2006–2100 and English (Languages)

PubMed

Date of the search: 1/10/2024

Set	Query
#1	"neotame" [Supplementary Concept] OR Neotame[tiab] OR E961[tiab] OR "E 961"[tiab] OR "165450–17-9"[tiab]
#2	("2006/01/01"[Date - Publication]: "3000"[Date - Publication]) AND ("neotame" [Supplementary Concept] OR Neotame[tiab] OR E961[tiab] OR "E 961"[tiab] OR "165450–17-9"[tiab])
#3	#2 Filters: English

Scifinder-n

Date of the search: 1/10/204

Query
Substance searched: "165450–17-9" > References Filters: Language – English Document type - Journal, review, clinical trial, preprint Date range: 2006–2024

The final number of references that were screened, after removal of duplicates (based on title, year, author, journal, volume, issue and page numbers) was 664 results.

For step **1.11.1** (Screening of the studies for relevance), the general principles reported in the protocol applied). 167 papers were included at the level of Title/abstract screening, whereas 497 papers were excluded. From the 167 papers included at the level of Title/abstract screening, 16 were selected for the full text screening and considered as possibly relevant, whereas 149 were either excluded at the level of full text screening, or categorised into technical data (57), exposure

[24]To cover the period from the latest EFSA evaluation with an overlap of 1 year (i.e. 2006) until 2 October 2024.

data (20), environmental data (20) or toxicological reviews (20), and further screened for confirmation of their relevance (see Figure B.1 for PRISMA flow chart). The full list of excluded studies was recorded in the Distiller SR Tool.

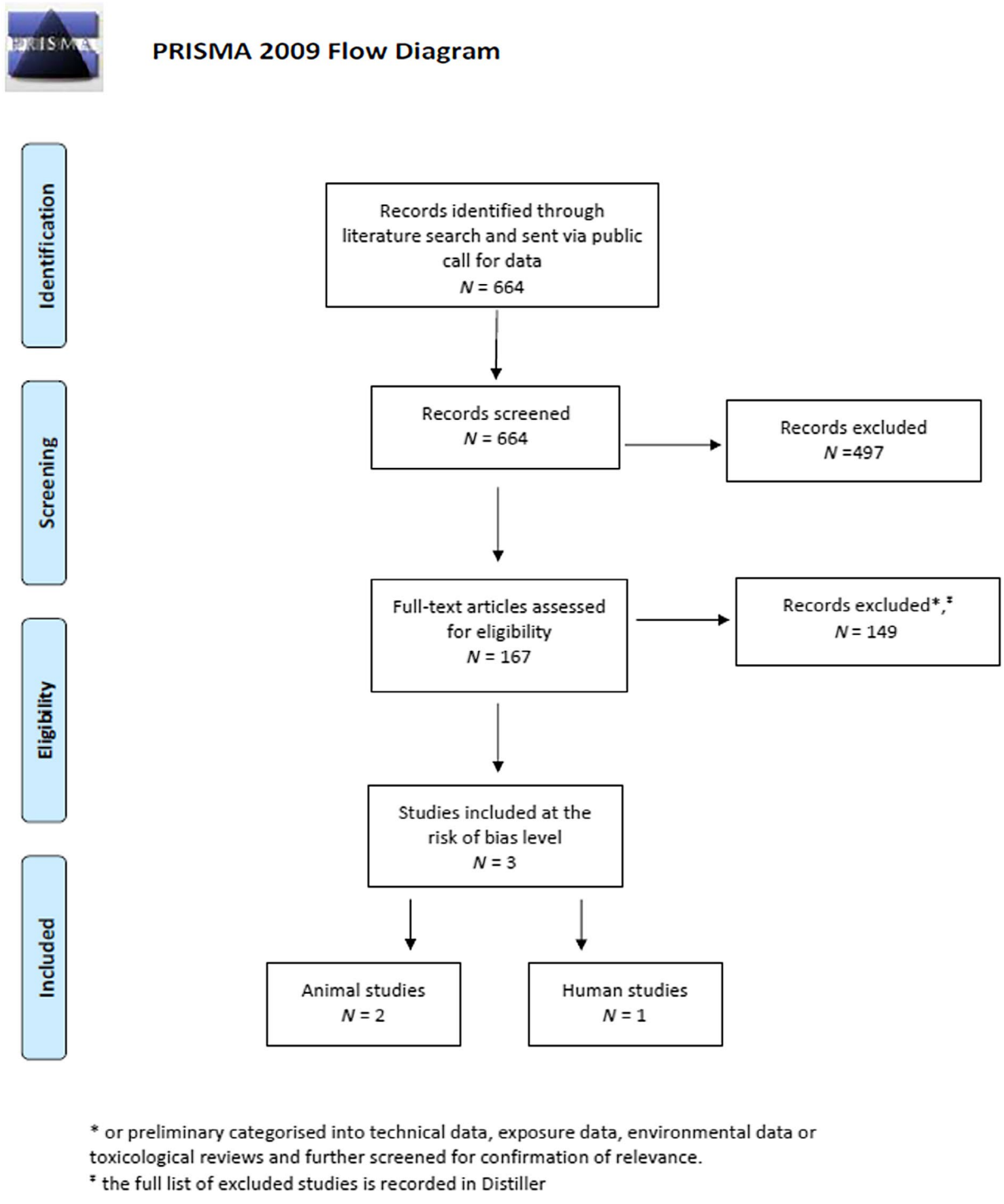


FIGURE B.1 PRISMA flow chart (adapted from Moher et al., 2009).

For step **1.12** (Evaluation of the risk of bias (RoB)), the criteria outlined in the protocol for the risk of bias evaluation of studies have been applied (NTP-OHAT, 2019) and the results of the RoB evaluation of the studies are summarised in Table B.

The studies evaluated for the RoB were allocated to a Tier (from 1 to 3 corresponding to decreasing levels of internal validity[11]) based on the rules as reported in step **1.12** (Evaluation of the risk of bias).

The evaluation of the RoB was conducted in parallel independently by two reviewers and, in case a conflict on Tier allocation of a study was identified, ad hoc discussion between the reviewers took place prior to a final agreed Tier allocation that was reached by consensus.

The Tier allocation was based on the rules detailed in step **1.12** of the protocol, and was automatically generated by the DistillerSR tool, after input of the ratings for the individual elements of the study considered for the RoB. At the end of the evaluation, the reviewers were requested to express their agreement or disagreement on the Tier generated by the tool

based on their expert judgement. No disagreements were identified. In case of disagreement, a clear justification should have been provided (see last two columns on the right of Table B).

As prescribed by the protocol (EFSA, 2020a), the study on which the derivation of the acceptable daily intake (ADI) was based was included in this risk assessment and evaluated according to the protocol together with any relevant literature published since the previous evaluation by SCF or EFSA, allowing 1 year of overlap (Tables B.1 and B.2).

TABLE B.1 Results of the RoB evaluation of the animal studies.

RefID	Authors and year	Study type	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Experimental conditions (performance bias)	Blinding of research personnel (performance bias)	Incomplete outcome data (attrition bias)	Confidence in exposure characterisation (detection bias)	Confidence in the outcome of the assessment (detection bias)	Selective reporting (reporting bias)	Appropriateness of statistical methods (other source of bias)	Tier (based on the rules)	Tier (agreement with automatically generated tier)
343	Zhu et al. (2016) (Third experiment)	Short-term toxicity	- -	+	++	-	+	++ +	- ++	++	++	3	Yes
724	Pivotal study (52-week dog study, cited in EFSA, 2007 as PCR 1017)	Long-term toxicity	++ +	+	++	-	++	++	++	++	++	2	Yes

Notes: RoB scoring: definitely low risk of bias (++), probably low risk of bias (+), probably high risk of bias (-), definitely high risk of bias (--). Split cells reporting two different scorings for the same risk of bias question express the view of the two independent reviewers.

Tier grading: Tier 1 (All the key questions are scored +/++ AND no more than one non-key question is scored – AND no non-key question is scored --; Tier 2 (All the other combinations not falling under tier 1 or 3); Tier 3: Any question is scored – OR more than one key question is scored –.

Questions: key questions (kQ): 1,3,4,6,7; non-key (nkQ): 2,5,8,9. kQ1: Random sequence generation (selection bias). kQ3: Experimental conditions (Performance bias). kQ4: Blinding of research personnel (Performance bias). kQ6: Confidence in exposure characterisation (detection bias). kQ7: Confidence in the outcome of the assessment (detection bias). nkQ2: Allocation concealment (selection bias). nkQ5: Incomplete outcome data (attrition bias). nkQ8: Selective reporting (reporting bias). nkQ9: Appropriateness of statistical methods (other source of bias).

TABLE B.2 Results of the RoB evaluation of human studies.

RefID	Authors and year	Study type	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Study population comparison	Confounding variables	Blinding of participants and personnel (	Incomplete outcome data (attrition bias)	Confidence in exposure to the interventions (detection bias)	Confidence in outcome of the assessment (detection bias)	Selective reporting (selection bias)	Appropriateness of statistical methods (other source of bias)	Tier (based on the rules)	Tier (agreement with automatically generated tier)
800	Gibbons et al. (2024)	Randomised control trial	+ ++	+			++	+	-	++	++	+ ++	2	Yes

Notes: RoB scoring: definitely low risk of bias (++), probably low risk of bias (+), probably high risk of bias (-), definitely high risk of bias (--). Split cells reporting two different scorings for the same risk of bias question express the view of the two independent reviewers.

Tier grading: Tier 1 (All the key questions are scored +/++ AND no more than one non-key question is scored – AND no non-key question is scored --; Tier 2 (All the other combinations not falling under tier 1 or 3); Tier 3: Any question is scored – OR more than one key question is scored –.

For RCT (randomised control trial), eight questions apply (Q 1, 2, 5–10). Four questions are considered key (kQ) (Q 1, 6, 7 and 8) and four non-key questions (nkQ) (2, 5, 9, 10). kQ1: Random sequence generation (selection bias). kQ6: Incomplete outcome data (attrition bias). kQ7: Confidence in exposure to the interventions (detection bias). kQ8: Confidence in outcome assessment (detection bias). nkQ2: Allocation concealment (selection bias). nkQ5: Blinding of participants and personnel (selection bias). nkQ9: Selective reporting (selection bias). nkQ10: Appropriateness of statistical methods (other source of bias).

In the case of neotame, the pivotal study (i.e. the 52-week dog study, cited in EFSA, 2007 as PCR 1017) was allocated to Tier 2. Therefore, the Panel considered appropriate rely on previous opinion (EFSA, 2007) and conclusion and do not assess the RoB for studies already considered, although received through the interested business operators.

Steps from 1.13 to 1.15 of the protocol were not applicable as no new biological and toxicological data that addressed sub-questions 3a and 3b were available.

APPENDIX C

Amount of methanol and formaldehyde released from neotame

Neotame is a monomethyl ester (Figure 1) with a molecular weight of 378 and so full hydrolysis of neotame to the metabolite NC-00751 would release methanol (MW 32) at 8.5% by weight (i.e. 32/378). The percentage of the dose which is metabolised to NC-00751 in humans is 100% which is the sum of NC-00751 found in urine and in faeces. Neotame is absorbed to only 34.4% of the dose and becomes systemically available in humans; for this calculation the Panel assumed that methanol which is formed by metabolism of the full dose of neotame in the gastrointestinal tract will be absorbed and systemically available. Therefore, the amount of methanol formed from a neotame dose of 10 mg/kg bw per day (proposed ADI) is 0.0.85 mg/kg bw per day. Hence, the maximum plasma steady state concentration of methanol that could arise from neotame-derived methanol can be calculated using the following equation:

$$\mathrm{Css} = f \times D / \left[\mathrm{Cl} \times \mathrm{tau}\right]$$

where:
Css = plasma steady state concentration
f = fraction absorbed (= 1.0 for methanol)
D = dose (0.85 mg/kg bw, at 70 kg bw = 59.9 mg = 1872 μmoles.
Cl = total body clearance (= 0.500 L/min)
tau = dosing interval (= 1 day = 1440 min)

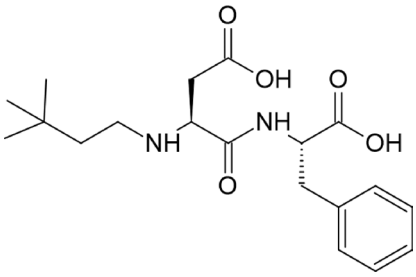
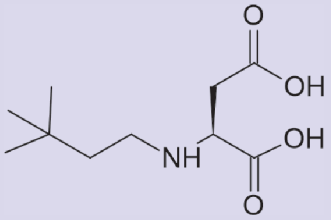
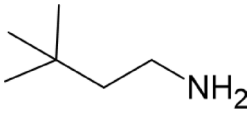
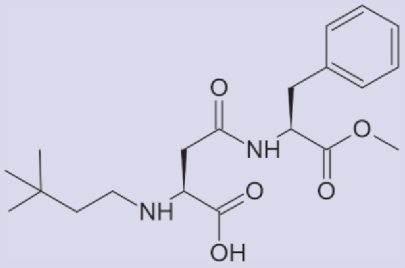
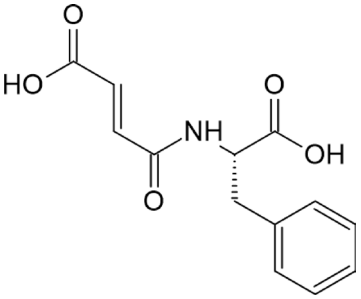
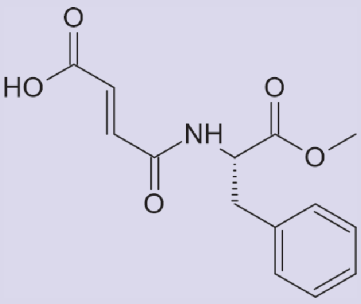
Using this approach, the maximum plasma steady state concentration of methanol that could arise from neotame-derived methanol is calculated to be 2.85 μM. Peak concentrations of methanol after three divided doses of neotame at the proposed ADI of 10 mg/kg bw are calculated to be about 5.4 μM.

Methanol is oxidised sequentially to formaldehyde which is further metabolised. Assuming that all methanol is converted to formaldehyde, 3.24 μM methanol could therefore produce a maximal steady state concentration of 3.24 μM formaldehyde/formaldehyde acetal. The increase in formaldehyde acetal associated with an exposure to neotame at the ADI (and ingested as one single dose) even if all methanol hydrolysed from neotame-containing food and formed as neotame metabolite after absorption of neotame, was converted to formaldehyde which was then converted to formaldehyde acetal would be less than 0.8% (steady state level) and less than 2.8% (peak level) of the normal intracellular endogenous levels. Such an additional burden can be evaluated in the light of naturally occurring variability. Kleinnijenhuis et al. (2013) reported that the coefficient of variation is 30% for the endogenous formaldehyde concentration in rat blood. Compared to this 30% variation, an increase of less than 0.8% (steady state level) and 3.7% (peak level) can be judged as being negligible given the fact that worst-case assumptions have been made in the scenario, for example, not taking into account decreasing formaldehyde concentrations due to metabolism and excretion.

APPENDIX D

Structurally related impurities/degradation products of neotame (E 961)

TABLE D.1 Possible impurities/degradation products of neotame (E 961).

Chemical name	Structure
N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751)	
N-[N-(3,3-dimethylbutyl)-L-aspartic acid (NC-00754)	
3,3-Dimethylbutylamine (NC-00759)	
N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine 1-methyl ester (NC-00764)	
Fumarylphenylalanine (NC-00767)	
N-Fumarylphenylalanine 1-methyl ester (NC-00768)	

(Continues)

TABLE D.1 (Continued)

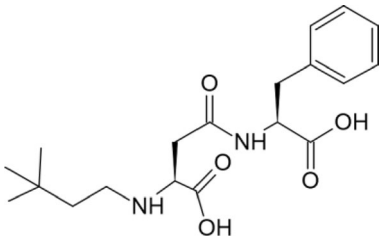
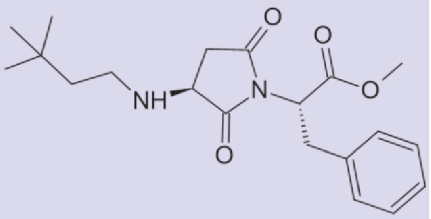
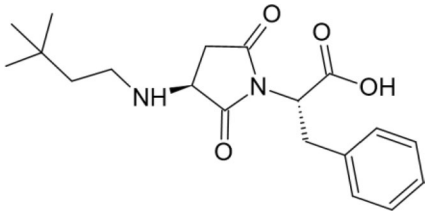
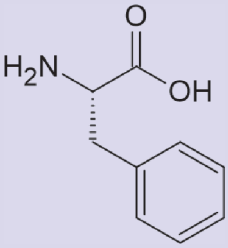
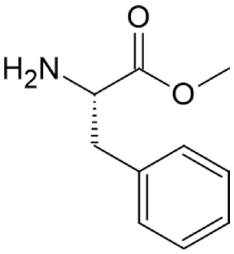
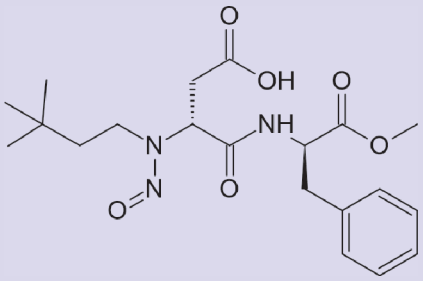
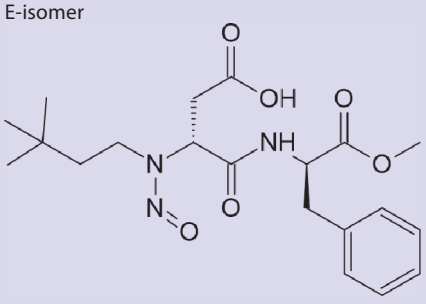
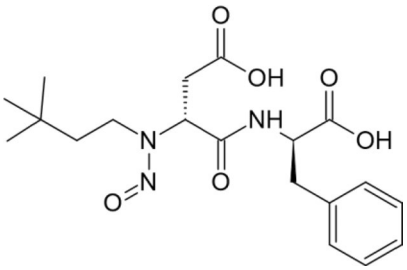
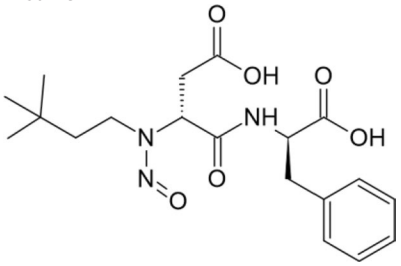
Chemical name	Structure
N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine (NC-00769)	
N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine 1-methyl ester (NC-00777)	
N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine (NC-00779)	
L-Phenylalanine	
L-Phenylalanine methyl ester	
N-nitroso-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methylester (NC-00799)	 E-isomer  Z-isomer

TABLE D.1 (Continued)

Chemical name	Structure
N-nitroso-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine (NC-00800)	 E-isomer  Z-isomer

APPENDIX E

Exposure calculations to organic impurities from the use of neotame (E 961)

The Panel considered the TTC approach to conduct a risk assessment for the impurities 'other related substances', given their lack of genotoxic potential.

All of the impurities included in the 'other related substances' (Appendix D, with the exception of L-phenylalanine and 3,3-dimethylbutylamine (Cramer Class I)) were classified into Cramer Class III (Cramer et al., 1978) (Appendix F, Table F.2), for which a TTC of 90 μg/person per day or 1.5 μg/kg bw per day is applicable provided that the substance is not genotoxic (EFSA Scientific Committee, 2019).

Exposure to the additive itself has been estimated to be 0.05 and 0.16 mg/kg bw per day, for the mean and the 95th percentile respectively. For the estimated exposure to the 'other related substances' from the use of E 961 to not raise a concern (less than the TTC value of 1.5 μg/kg bw per day), their presence should be less than 0.9% in the food additive. The Panel noted that the reported analytical results for the impurities in total (0.05%–0.19% in 10 samples) are lower than the limit of 0.9% and even by considering them collectively, the potential exposure does not exceed the Cramer Class III value of 1.5 μg/kg bw per day (0.10 and 0.32 μg/kg bw per day) (Table E.1).

Considering the exposure estimates, the purity value of neotame and the analytical data provided for the 'other related substances', the Panel noted that no concern is identified for those impurities and thus, there is no need for them to be included in the EU specifications.

TABLE E.1 Potential exposure (μg/kg bw per day) to organic impurities.

Exposure to E 961 (mg/kg bw per day)	Scenario based on the levels of impurities at the rounded highest measured values provided by one IBO (Documentation provided to EFSA n. 1, 3) Exposure to 'other related substances' at a concentration of 0.2% in the additive (μg/kg bw per day)
0.05[a]	0.10
0.16[b]	0.32

[a]Highest exposure level among the different population groups (refined brand-loyal scenario – toddlers – mean) (Table 6).

[b]Highest exposure level among the different population groups (refined brand-loyal scenario scenario – toddlers – 95th percentile) (Table 6).

APPENDIX F

In silico assessment of neotame (E 961) impurities

The genotoxicity and Cramer class of the impurities/degradation products of neotame (E961) described in Appendix D were evaluated using in silico tools. A suite of VEGA models, and the rule-based OECD QSAR Toolbox profilers were interrogated (Table F.1).

Name and code of analysed compounds

- Compound 1 – N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine (NC-00751
- Compound 2 – N-[N-(3,3-dimethylbutyl)-L-aspartic acid (NC-00754)
- Compound 3 – 3,3-dimethylbutylamine (NC-00759)
- Compound 4 – N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine 1-methyl ester (NC-00764)
- Compound 5 – Fumarylphenylalanine (NC-00767)
- Compound 6 – N-Fumarylphenylalanine 1-methyl ester (NC-00768)
- Compound 7 – N-[N-(3,3-dimethylbutyl)-L-β-aspartyl]-L-phenylalanine (NC-00769)
- Compound 8 – N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine 1-methyl ester (NC-00777)
- Compound 9 – N-[N-(3,3-dimethylbutyl)-L-aspartamidyl]-L-phenylalanine (NC-00779)
- Compound 10 – L-Phenylalanine (Phe)
- Compound 11 – L-Phenylalanine methyl ester
- Compound 12 – N-nitroso-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine methylester (NC-00799)
- Compound 13 – nitroso-(3,3-dimethylbutyl)-L-aspartyl-L-phenylalanine (NC-00800)
- Compound 14 – N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester (neotame, E961)

TABLE F.1 Summary of results. VEGA models.

	NC-00751 (1)	NC-00754 (2)	NC-00759 (3)	NC-00764 (4)	NC-00767 (5)	NC-00768 (6)	NC-00769 (7)	NC-00777 (8)	NC-00779 (9)	Phe (10)	Phe methyl ester (11)	NC-00799 (12)	NC-00800 (13)	E 961 (14)
Ames consensus 1.0.4	**NM** (0.825)	**NM** (0.65)	**NM** (0.825)	**NM** (0.825)	**NM** (0.35)	**NM** (0.2)	**NM** (0.825)	**NM** (0.825)	**NM** (0.725)	**NM** (1.0)	**NM** (0.9)	**NM** (0.225)	**NM** (0.225)	**NM** (0.825)
Ames CAESAR 2.1.14	**NM/G**	**NM/G**	**NM/G**	**NM/G**	NM/M[2,3]	M/L[2,3]	**NM/G**	**NM/G**	**NM/G**	**NM/G**	**NM/G**	M/L[2,7]	M/L[2,7]	**NM/G**
Ames ISS 1.0.3	NM/M[1]	NM/L[2]	NM/M[3]	NM/M[3]	M/L[1,2,5]	M/L[2,5,6]	NM/M[1]	NM/M[3]	NM/L[1,2]	**NM/G**	**NM/G**	M/L[1,2,3]	M/L[1,2,3]	NM/M[3]
Ames SarPy-IRFMN 1.0.8	**NM/G**	**NM/G**	**NM/G**	**NM/G**	NM/M[1,2]	NM/M[2]	**NM/G**	**NM/G**	**NM/G**	**NM/E**	**NM/G**	M/L[2]	M/L[2]	**NM/G**
Ames KNN-Read-across 1.0.1	**NM/G**	NM/M[1]	**NM/G**	**NM/G**	NM/L[6]	NM/L[2,6]	**NM/G**	**NM/G**	**NM/G**	**NM/E**	**NM/G**	**NM/G**	**NM/G**	**NM/G**
CAs CORAL 1.0.1	I/M[3]	I/M[1]	I/M[2]	I/M[2,3]	A/M[2,3]	A/M[2,3]	I/M[2,3]	I/M[1,2,3]	I/M[2]	A/L[2]	A/L[2]	**I/G**	**I/G**	I/M[2,3]

(Continues)

TABLE F.1 (Continued)

	NC-00751 (1)	NC-00754 (2)	NC-00759 (3)	NC-00764 (4)	NC-00767 (5)	NC-00768 (6)	NC-00769 (7)	NC-00777 (8)	NC-00779 (9)	Phe (10)	Phe methyl ester (11)	NC-00799 (12)	NC-00800 (13)	E 961 (14)
In vitro MN IRFMN-VEERMER 1.0.1	A/M[1,2]	A/M[1,2]	**I/G**	**A/G**[3]	A/M[2,3]	A/M[2,3]	A/M[2,3]	A/M[2,3]	A/M/[2,3]	A/M[1,2]	A/M[2,6]	**A/G**[3]	**A/G**[3]	**A/G**[3]
In vivo MN IRFMN 1.0.2	I/M[2]	I/M[2]	I/M[2]	**I/G**[2]	A/M[2]	I/L[4]	I/M[2]	NP	NP	I/M[2]	I/M[2]	I/M[2]	I/M[2]	I/M[2]
Cramer TOXTREE 1.0.1	Class I[4]	Class III[4]	Class I[4]	Class I[4]	Class I[4]	Class I[4]	Class I[4]	Class III[4]	Class III[4]	Class I[4]	Class I[4]	Class III[4]	Class III[4]	Class I[4]

In the table, prediction/reliability codes. In bold the predictions with good reliability. Only predictions with good/moderate reliability are considered. Code of predictions: NM: non-mutagenic; M: mutagenic; I: inactive; A: active; NP: not predicted. Reliability code: G: good reliability (The predicted compound is into the Applicability Domain of the model); M: moderate reliability (The predicted compound could be out of the Applicability Domain of the model); L: low reliability (The predicted compound is outside the Applicability Domain of the model); E: experimental data available.

[1]Accuracy of prediction for similar molecules found in the training set is not optimal.

[2]Similar molecules found in the training set have experimental values that disagree with the predicted value.

[3]Only moderately similar compounds with known experimental value in the training set have been found.

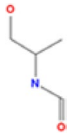
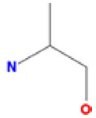
[4]Impossible to perform an assessment (unable to perform the applicability domain check).

[5]The following alerts have been found: SA10 alfa, beta unsaturated carbonyls.

[6]Accuracy of prediction for similar molecules found in the training set is not adequate.

[7]The following relevant fragments have been found: SA21 Alkyl and aryl N nitroso groups.

Alerting fragments for activity in the in vitro MN assay (IRFMN-VERMEER 1.0.1)

Alerting fragment	Structure	Compound
#1		1, 4–9, 12–14
#18		4, 6, 8, 11, 12, 14
#43		1–9, 12–14

(Continued)

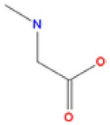
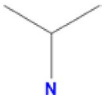
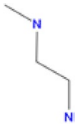
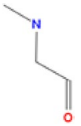
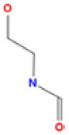
Alerting fragment	Structure	Compound
#76		1–9, 12–14
#87		1–11, 14
#92		1–14
#95		1, 3, 7–9, 12–14
#108		1–9, 12–14
#119		1, 3–9, 12–14

TABLE F.2 Summary results. OECD QSAR ToolBox.

	NC-00751 (1)	NC-00754 (2)	NC-00759 (3)	NC-00764 (4)	NC-00767 (5)	NC-00768 (6)	NC-00769 (7)	NC-00777 (8)	NC-00779 (9)	Phe (10)	Phe methyl ester (11)	NC-00799 (12)	NC-00800 (13)	E 961 (14)
Carcinog. Genotox/ non-genotox by ISS	NA	NA	NA	NA	Alpha-beta unsaturated carbonyl	Alpha-beta unsaturated carbonyl	NA	NA	NA	NA	NA	Alkyl N-nitroso groups	Alkyl N-nitroso groups	NA
Ames, CA, MN vit by OASIS	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	SN1[2]	SN1[2]	NA

(Continues)

TABLE F.2 (Continued)

	NC-00751 (1)	NC-00754 (2)	NC-00759 (3)	NC-00764 (4)	NC-00767 (5)	NC-00768 (6)	NC-00769 (7)	NC-00777 (8)	NC-00779 (9)	Phe (10)	Phe methyl ester (11)	NC-00799 (12)	NC-00800 (13)	E 961 (14)
Ames test alert by ISS	NA	NA	NA	NA	Alpha-beta unsaturated carbonyl	Alpha-beta unsaturated carbonyl	NA	NA	NA	NA	NA	Alkyl N-nitroso groups	Alkyl N-nitroso groups	NA
DNA binding by OASIS	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	SN1[2]	SN1[2]	NA
DNA binding by OECD	Michael addition[1]	NA	NA	Michael addition[1]	Michael addition[1]	Michael addition[1]	Michael addition[1]	Michael addition[1]	Michael addition[1]	Michael addition[1]	Michael addition[1]	Michael addition[1]	Michael addition[1]	Michael addition[1]
Protein binding alerts for chromosomal aberrations by OASIS	NA	NA	NA	NA	AN2[3]	AN2[3]	NA	NA	NA	NA	NA	NA	NA	NA
Toxic hazard classification by Cramer	Class III	Class III	Class I	Class III	Class III	Class III	Class III	Class III	Class III	Class I	Class III	Class III	Class III	Class III

Abbreviation: NA, no alerts found.

[1]Michael addition >> P450 Mediated Activation to Quinones and Quinone-type Chemicals.

[2]SN1 > Nucleophilic attack after nitrosonium cation formation.

[3]AN2 >> Michael addition to activated double bond.

APPENDIX G

Benchmark dose analysis

In vitro micronucleus (MN) data submitted by the IBO (Documentation provided to EFSA n. 4) were analysed using the EFSA 'Bayesian BMD' webtool.[25] For BMD analyses, the Panel converted in vitro MN assay data from %MN to the frequency of MN observed per 1000 cells, a binary variable which can be modelled using quantal models to estimate the BMD.

The EFSA guidance on BMD modelling (EFSA Scientific Committee, 2022) does not provide formal guidance on the modelling of data derived from in vitro MN assays, in particular the selection of the appropriate benchmark response (BMR). In the scientific literature (e.g. Powley et al., 2024, Kuo et al., 2022, Zeller et al., 2017, Soeteman-Hdernandez et al., 2015), for MN data, several BMRs were suggested including the use of percentage-based BMRs, BMR values derived from the standard deviation (SD) of within-assay control values, or BMR values based on historical negative control data.

To describe the biological and experimental variability of the test system for this analysis, the Panel considered historical control data to represent a better comparator than the concurrent control data which are affected by uncontrolled experimental variation.

According to OECD test guideline 487,[26] provided all acceptability criteria are met, a test chemical is considered positive in the in vitro MN test if any of the tested concentrations show a statistically significant increase compared with the concurrent negative controls. Guideline 487 also states that concurrent in vitro MN assay controls should ideally be within the 95% control limits of the distribution of the laboratory's historical negative control database.

Taking into account the information provided in the scientific literature, the acceptability criteria for in vitro MN assays outlined in the OECD guideline 487, and expert judgement, for the BMD modelling, the FAF Panel chose to use BMRs corresponding to the 95th quantile (from estimated Beta distribution) of the historical negative control data ($n = 40$) reported by the IBO (corresponding to BMRs of 107% and 150% in the 4 and 24 h MN assays, respectively). Deriving a BMR based on the 95th quantile of the estimated distribution based on the historical control data of the laboratory performing the MN assays, leaves only a 5% chance that a value above the specified 95th quantile lies inside the control range. The 5% chance level is a commonly used statistical cut-off, for instance when defining significant effect p-values < 0.05 are considered significantly different; reflecting a 5% risk of type I error, in terms of rejecting a null hypothesis, while the null hypothesis is actually true.

In the following sections a detailed account of the data used and the analyses performed is presented.

G.1 | Data description

The endpoint to be analysed was the number of binucleated cells obtained in an in vitro micronucleus (MN) assay with neotame. The data set selected for the analysis consisted of the results obtained after 4- and 24 h treatment in the absence of metabolic activation. A description of the rationale for the selection of the data to be used in the BMD analysis is provided in Section 3.5.2.

4 h MN assay

TABLE G.1 4 h micronucleus (MN) assay without metabolic (S9) activation. Count of binucleated cells expressed in percent (%MN); number of binucleated cells (NBC) per number of cells (NC).

Group	Concentration	%MN	NBC	NC
Negative control (DMSO)	0	0.3	*3*	1000
	0	0.6	*6*	1000
	0	0.8	*8*	1000
	0	0.1	*1*	1000
Neotame	500	1.7	17	1000
	500	2	20	1000
	1000	2.8	28	1000
	1000	2.1	21	1000
	1200	2.6	26	1000
	1200	2.4	24	1000
Positive control		6.2	62	1000
		8.7	87	1000

[25] https://r4eu.efsa.europa.eu/app/bmdbayesian.

[26] https://www.oecd.org/en/publications/test-no-487-in-vitro-mammalian-cell-micronucleus-test_9789264264861-en.html.

24 h MN assay

TABLE G.2 24 h micronucleus (MN) assay without metabolic (S9) activation. Count of binucleated cells expressed in percent (%MN); number of binucleated cells (NBC) per number of cells (NC).

Group	Concentration	%MN	NBC	NC
Negative control (DMSO)	0	0	0	1000
	0	0.1	1	1000
	0	0.3	3	1000
	0	0.5	5	1000
Neotame	250	0.8	8	1000
	250	0.3	3	1000
	350	1.9	19	1000
	350	2.3	23	1000
	400	3.9	39	1000
	400	2.7	27	1000
	450	2.9	29	1000
	450	3.2	32	1000
Positive control		3.8	38	1000
		2.4	24	1000

G.2 | Software used

Calculations and data conversions were performed using R (Annex A); data were displayed and summarised in Microsoft Excel (Annex C). BMD modelling results were obtained using the EFSA webtool for Bayesian BMD analysis, which uses the R-package [BMABMDR] version 0.1.5 for the underlying calculations.

G.3 | Justification of any deviation from the procedure and assumptions

The current BMD implementation is not designed to model data expressed as percentages, i.e.? following a Beta distribution. Data were converted from %MN to the frequency of binucleated cells (NBC) per number of cells (NC), which is a binary variable and can be modelled using quantal models to estimate BMD.

TABLE G.3 4 h micronucleus assay. Count of binucleated cells expressed as number of binucleated cells (NBC) per number of cells (NC).

Concentration (μg/mL)	NBC	NC
0	18	4000
500	37	2000[a]
1000	49	2000[a]
1200	50	2000[a]

[a]Statistically significantly different form control group.

TABLE G.4 24 h micronucleus assay. Count of binucleated cells expressed as number of binucleated cells (NBC) per number of cells (NC).

Concentration (μg/mL)	NBC	NC
0	9	4000
250	11	2000[a]
350	42	2000[a]
400	66	2000[a]
450	61	2000[a]

[a]Statistically significantly different form control group.

Considering that the frequencies of MN cells observed are rather small, the extra risk definition of the BMD[27] has been adjusted to ensure that BMD definition does not correspond to the added risk, and so the relative definition used for continuous outcomes was the preferred option in this situation. Considering that the implementation only provides the extra risk definition, derivations were used to define the corresponding extra risk BMR that align with the desired relative definition (Equations G.1–G.3).

$$BMR_C = \frac{\pi(BMD) - \pi(0)}{\pi(0)}$$

$$BMR_Q = \frac{\pi(BMD) - \pi(0)}{1 - \pi(0)}$$

$$BMR_Q = BMR_C \times \frac{\pi(0)}{1 - \pi(0)} = \frac{\pi(BMD) - \pi(0)}{\pi(0)} \times \frac{\pi(0)}{1 - \pi(0)} = \frac{\pi(BMD) - \pi(0)}{1 - \pi(0)}$$

Equations G.1–G.3: Definition for BMR for continuous data (BMRc) and calculation of BMR for quantal data (BMRq). The letter π denominates the probability of an event.

To define the BMRs relating to historic control values reported by the IBO, the following data were used.

TABLE G.5 Historic control data ($n = 40$) of the 4 h MN assay without metabolic activation. Count of binucleated cells expressed in percent (%MN); number of binucleated cells (NBC) per number of cells (NC).

	%MN	NBC	NC
Min	0.13	1.3	1000
Max	0.78	7.8	1000
Mean	0.4	4	1000
SD	0.16	1.6	1000
95%CI_L	0.08	0.8	1000
95%CI_U	0.72	7.2	1000

TABLE G.6 Historic control data ($n = 40$) of the 4 h MN assay without metabolic activation. Historic control data ($n = 40$); 24 h MN assay. Count of binucleated cells expressed in percent (%MN); number of binucleated cells (NBC) per number of cells (NC).

	%MN	NBC	NC
Min	0.03	0.3	1000
Max	1	10	1000
Mean	0.35	3.5	1000
SD	0.17	1.7	1000
95%CI_L	0.01	0.1	1000
95%CI_U	0.69	6.9	1000

Based on the reported means ($\hat{\pi}$) and standard deviations ($\hat{sd}$) of the 4 and 24 h historic control data, alpha (α) and beta (β) parameters of the Beta distribution to represent the probability of observing micronucleate cells were calculated, considering the following:

$$\hat{\pi} = \frac{\alpha}{\alpha + \beta}$$

$$\hat{sd} = \sqrt{\frac{\alpha \bullet \beta}{(\alpha+\beta)^2 \bullet (\alpha + \beta + 1)}}$$

[27]The EFSA Guidance on the use of the benchmark dose approach in risk assessment (REF), defines extra risk as the additional risk (incidence at a given dose, π(BMD), minus incidence in the controls, π(0)) divided by the non-affected fraction of the control group, 1-π(0).

Then solving this equation we can obtain the parameters α and β for the corresponding Beta distribution. Following parameter estimation, the modes and means of the Beta distribution were derived to be compared with the observed values from the historical control data. Probability calculations were performed to determine the likelihood of observing values outside of historical control values (observed minimum and maximum proportions, as well the probability of being outside the 95th CI ranges provided). BMRs were derived as the ratio of the difference between τ (defining a potential threshold that it could be considered outside the control ranges) and μ (the most likely value of the Beta distribution) to μ, the figure below illustrates this concept, presenting the Beta distribution and the quantities mentioned (μ and τ) (Figure G.1).

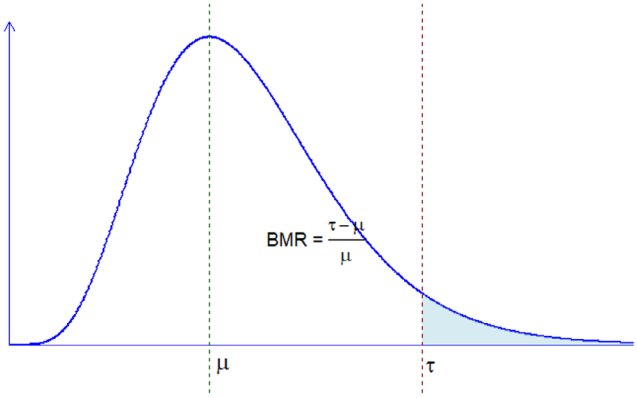


FIGURE G.1 Illustration of the Beta distribution, the most likely value μ and a hypothetical threshold value τ that represents specific quantile of the distribution, considering the blue area to be small probability to be above such threshold.

G.4 | Results

BMC (BMCL – BMCU) values for BMRs corresponding to the 95th quantile are provided. The detailed analyses report outputs from the EFSA 'Bayesian BMD' webtool can be found in Annex D (4 h) and Annex E (24 h).

	4 h MN	24 h MN
BMR (95th qntBeta)	*1.065272*	1.497662
BMRQ (CES)	0.00482	0.00338
BMCL	44.2	155.7
BMC	**200.3**	**211.3**
BMCU	364.6	249.9

G.5 | Conclusions

For the 4 h MN assay (without metabolic activation), a BMC (BMCL-BMCU) of 200 (44–365) mg/L was obtained; for the 24 h MN assay (without metabolic activation) a BMC (BMCL-BMCU) of 211 (156–250) mg/L was obtained.

ANNEXES

Annexes A–E can be found in the online version of this output ('supporting information' section).
Annex A: Exposure data and estimates.
Annex B: R script for BMD analysis.
Annex C: Excel file summary BMD analysis.
Annex D: BMD report 4 h in vitro MN assay (without metabolic activation).
Annex E: BMD report 24 h in vitro MN assay.