Adopted: 22 October 2025

DOI: 10.2903/j.efsa.2025.9738

efsa JOURNAL

SCIENTIFIC OPINION

Safety evaluation of jagua (genipin-glycine) blue as a food additive

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Abstract

The EFSA Panel on Food Additives and Flavourings (FAF Panel) provides a scientific opinion on the safety of jagua (genipin-glycine) blue as a new food additive. Jagua (genipin-glycine) blue is obtained by water extraction of the ground pulp of the peeled, unripe fruits of *Genipa americana* L. and is the result of a reaction between genipin (iridoid present in the fruit) and externally added glycine. This reaction leads to the formation of a blue-coloured polymer and minor colouring components. In vitro Caco-2 cell permeability test demonstrated a low permeability of jagua (genipin-glycine) blue, but repeated dose toxicity studies showed organs discoloration and green-coloured urine, demonstrating some absorption. The toxicological data set comprised acute, sub-chronic toxicity, genotoxicity studies and also a 12-month toxicity study including in utero exposure. Jagua (genipin-glycine) blue was not genotoxic, and no adverse effects were observed in the repeated dose toxicity studies up to the highest doses tested. The Panel derived an acceptable daily intake (ADI) of 34 mg/kg bw per day or 12 mg/kg bw per day expressed as blue polymer, based on a no observed adverse effect level (NOAEL) of 3385 mg/kg bw per day, the highest dose tested, from the 12-month toxicity study and an uncertainty factor of 100. At the proposed maximum use level exposure assessment scenario, the 95th percentile of exposure approximately ranged from 1 mg/kg bw per day in the elderly to 27 mg/kg bw per day in toddlers. The Panel noted that both the mean and 95th percentile estimates of exposure did not exceed the proposed ADI in all population groups. The same was true for the exposure to the blue polymer assuming a 40% content in the proposed food additive. The Panel concluded there is no safety concern for jagua (genipin-glycine) blue as a food additive at the proposed use and use levels.

KEYWORDS

blue-coloured polymer, food colour, *Genipa americana*, genipin, jagua blue

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SUMMARY

Following a request from the European Commission to the European Food Safety Authority (EFSA), the Panel on Food Additives and Flavourings (FAF) was asked to provide a scientific opinion on the safety of jagua (genipin-glycine) blue as a food additive in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

Jagua (genipin-glycine) blue is produced by water extraction of the ground pulp of the peeled, unripe fruits of *Genipa americana* L., followed by a reaction between an iridoid compound (genipin) and added glycine (E 640), forming a blue-coloured polymer (blue polymer, Compound 4) and minor colouring components (Compounds 1–3). The final product contains 20%–40% blue polymer, minor colouring compounds (< 0.4%), carbohydrates and proteins and is commercialised as a deep blue powder, containing a carrier (e.g. maltodextrin or modified starch). Residual genipin was below the limit of quantification (LOQ) (< 10 ppm).

The Panel reviewed the proposed specifications and recommended a more detailed description regarding the manufacturing process in the definition. In addition, the Panel considered that the proposed specification range of 140%–280% for the colour E10% parameter is wide, compared to the analytical data provided (243%–273%), while the proposed specification limits for total fat of < 10% and for yeasts and moulds of below 100 CFU/g are higher than the analytical data provided (0.31%–1.99% for total fat and < 10 CFU/g for yeasts and moulds). The Panel also noted that the total carbohydrate contents in the proposed food additive were calculated by the applicant by difference (100% – protein % – fat % – moisture % – ash %), without considering the functional component (blue polymer = 20% to 40%, according to the specifications), the minor blue colouring components and other components. Based on the data provided, the Panel considered that the water solubility of the blue polymer is at least 6 g/L and that it should be described as 'slightly soluble' in the proposed specifications.

Concerning toxic elements, analytical data on the levels of arsenic (As), lead (Pb), cadmium (Cd) and mercury (Hg) were provided. The Panel assessed the potential risk under two scenarios and concluded that the proposed specification limits should be lowered, particularly for inorganic arsenic (iAs), whose margin of exposure was close to 1.

With respect to the toxicological testing strategy, the Panel followed a conventional risk assessment as described in the 'Guidance for submission for food additive evaluation' (EFSA ANS Panel, 2012) since no concern was identified with regard to the potential presence of small particles, including nanoparticles of the blue polymer, at the proposed uses and use levels of the proposed food additive. Indeed, although the Panel noted that, based on the data provided, the presence of small particles including nanoparticles in jagua (genipin-glycine) blue as produced was confirmed, taking into account the reported solubility of the blue polymer, the maximum proposed use levels 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 the blue polymer is to be expected in foods and/or in the gastrointestinal tract and that ingested particles (if any) would not persist.

The Panel considered jagua (genipin-glycine) blue to be stable as a powder and in food matrices under the evaluated conditions.

Regarding absorption, in vitro Caco-2 cell permeability test showed low permeability of jagua (genipin-glycine) blue. However, in vivo studies in dogs and rats showed green-coloured urine and discoloration of some organs, suggesting that at least partial absorption of the proposed food additive occured. Given its high molecular weight (over 6000 Da), jagua (genipin-glycine) blue itself is expected to have low absorption, and therefore the Panel considered that the observed discoloration is likely due to low molecular weight components or breakdown products. Based on this, the Panel considered that some absorption of the proposed food additive has been demonstrated.

The toxicological data set comprised in vitro and in vivo genotoxicity studies, studies on acute toxicity, sub-chronic toxicity (90-day toxicity study in rat; 90-day toxicity study in dog) and a long-term toxicity study including in utero exposure (12-month dietary toxicity study in rat). Although no standard reproductive and developmental toxicity studies were provided, the 12-month dietary toxicity study including in utero exposure was considered sufficient by the Panel to cover these endpoints and to conclude on the safety of jagua (genipin-glycine) blue.

With respect to genotoxicity, taking into account the negative experimental data for jagua (genipin-glycine) blue and genipin, together with the quantitative structure – activity relationship model ((Q)SAR) analysis of low molecular weight components, the Panel concluded that the proposed food additive does not raise a safety concern for genotoxicity.

In rats, both the 28- and 90-day studies revealed no adverse effects up to the highest tested dose of 1000 mg/kg body weight (bw) per day. Minor findings such as dark discoloration of kidneys and testes were attributed to the intense blue colour of the test substance rather than toxicity. Similarly, in dogs, no treatment-related effects were observed in any clinical, haematological or histopathological parameters and the same no observed adverse effect level (NOAEL) of 1000 mg/kg bw per day was identified.

A 12-month dietary study in rats, including in utero exposure, also demonstrated no treatment-related systemic or organ toxicity. Occasional discoloration of kidneys and gastrointestinal segments was observed but considered non-adverse in the absence of histopathological alterations. Malignant lymphomas were observed in 2/25 males of the F1 generation. These were confirmed to be spontaneous and unrelated to treatment. Consequently, a NOAEL of 50,000 mg/kg diet (approximately 3400 mg/kg bw per day), the highest dose tested, was identified. Reproductive and developmental endpoints assessed within this study showed no adverse effects on fertility, gestation, litter parameters or offspring development.

Based on the available data, the Panel established an ADI of 34 mg jagua (genipin-glycine) blue/kg bw per day or 12 mg/kg bw per day expressed as blue polymer, based on a NOAEL of 3385 mg jagua (genipin-glycine) blue/kg bw per day (or 1232 mg blue polymer/kg bw per day, considering a blue polymer content of 36.4%) and applying the default uncertainty factor of 100.

Estimated dietary exposure to jagua (genipin-glycine) blue did not exceed the ADI in all populations groups, even at the proposed maximum use levels (up to 5000 mg/kg food). The highest exposure levels were observed in toddlers i.e. approximately 9 mg/kg bw per day at the mean and 27 mg/kg bw per day at the 95th percentile. The same was true for the exposure to the blue polymer assuming a 40% content in jagua (genipin-glycine) blue. Major contributors to the overall exposure included flavoured fermented milk products, breakfast cereals and edible ices.

Taking into account the exposure estimates, the Panel concluded there is no safety concern for jagua (genipin-glycine) blue as a food additive at the proposed uses and use levels.

1 | INTRODUCTION

The present scientific opinion deals with the safety evaluation of jagua (genipin-glycine) blue proposed as a new food additive (food colour) in a variety of food categories.

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

1.1.1 | Background

The use of food additives is regulated under the European Parliament and Council Regulation (EC) No 1333/2008[1] on food additives. Only food additives that are included in the Union list, in particular Annex II to that regulation, may be placed on the market and used in foods under conditions of use specified therein. Moreover, food additives shall comply with the specifications as referred to in Article 14 of that Regulation and laid down in Commission Regulation (EU) No 231/2012.[2]

An application has been introduced for the authorisation of the use of jagua (genipin-glycine) blue as a colour in several food categories of Annex II to Regulation (EC) No 1333/2008.

According to the applicant, jagua (genipin-glycine) blue is manufactured by water extraction of the ground pulp of the peeled unripe fruits of *G. americana* as the result of a reaction between genipin (an iridoid present in the fruit) and glycine. This naturally occurring reaction produces a mixture of blue compounds, which are also easily observable in the unripe fruits after being cut.

1.1.2 | Terms of Reference

The European Commission requests the European Food Safety Authority to perform a risk assessment to provide a scientific opinion on the safety of the proposed use of jagua blue as a food additive, in accordance with Regulation (EC) No. 1331/2008[3] establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

1.2 | Information on existing evaluations and authorisations

Jagua (genipin-glycine) blue was first evaluated as a food additive by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2017 at its 84th meeting. The Committee reviewed chemical, technical, biological and toxicological data, including unpublished in vitro studies, genotoxicity studies and in vivo repeated dose toxicity studies in rats and dogs, as well as dietary exposure data. No treatment-related adverse effects were found at the highest doses tested in 90-day toxicity studies (330 and 338 mg/kg bw per day, expressed on a 'blue polymer' basis, in the dog and rat, respectively); however, blue/green urine was observed in dogs together with an increase in serum bilirubin. Therefore, it was concluded that it could not be excluded that some low molecular weight components of the food additive were absorbed and excreted. JECFA expressed a concern regarding the potential toxicity of this fraction of jagua (genipin-glycine) blue. Therefore, JECFA recommended to perform additional biological and toxicological studies, using higher doses, to adequately investigate the low molecular weight fraction of the proposed food additive. In addition, the Committee required additional information on the characterisation of the test item, in particular of the low molecular weight components, a validated method for the determination of dimers and data on concentrations of dimers from five batches of the commercial product. Because of the limitations identified in the data available, the Committee was not able to finalise the assessment of this food additive and no acceptable daily intake (ADI) was established. Nevertheless, a tentative specifications monograph was published for jagua (genipin-glycine) blue (JECFA, 2017).

After receiving the requested data, the evaluation of jagua (genipin-glycine) blue was completed by JECFA in 2020 at its 89th meeting, when an ADI of 11 mg/kg bw per day based on blue polymer was established (JECFA, 2020). The ADI was set on the basis of the absence of treatment-related long-term toxicity, as well as reproductive and developmental toxicity, in a 12-month rat dietary study with in-utero exposure, identifying a no observed adverse effect level (NOAEL) of 1127 mg/kg bw per day of the blue polymer, corresponding to the highest dose tested. Regarding the dietary exposure, JECFA noted that the upper end of the high-level dietary exposure estimates for jagua (genipin-glycine) blue (expressed on 'blue polymer' basis) of 11.5 mg/kg bw per day for infants and toddlers was above the established ADI. However, considering the conservative nature of the dietary exposure assessments and the fact that the ADI was derived from a NOAEL corresponding to the highest dose tested, it was concluded that the exposure to jagua (genipin-glycine) blue did not represent a health

[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, pp. 16–33.

[2]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, pp. 1–295.

[3]Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, pp. 1–6.

concern. The specifications for jagua (genipin-glycine) blue were revised and the tentative status of the specifications was removed.

At the time of the submission of this dossier, the applicant claimed that additional registration processes for the use of jagua (genipin-glycine) blue as a food additive were currently in progress in other countries of interest outside Europe (e.g. the United States, Mexico and Brazil). Currently, jagua (genipin-glycine) blue has been authorised as a food colour in the USA,[4] Argentina, Brazil, Paraguay and Uruguay.[5]

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier to support the safety evaluation of jagua (genipin-glycine) blue proposed as a food additive in a variety of food categories (Documentation provided to EFSA No. 1).

Following several requests for additional information sent by EFSA, the applicant provided additional data on 10/8/2022 (Documentation provided to EFSA No. 2), on 9/12/2022 (Documentation provided to EFSA No. 3), on 15/6/2023 (Documentation provided to EFSA No. 4), on 23/6/2023 (Documentation provided to EFSA No. 5), on 26/4/2024 (Documentation provided to EFSA No. 6), on 27/8/2025 and on 2 and 3/10/2025 (Documentation provided to EFSA No. 7, 8).

2.2 | Methodologies

This opinion was formulated following the principles described in the EFSA Guidance of the Scientific Committee 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.

The current 'Guidance for submission for food additive evaluation' (EFSA ANS Panel, 2012), the Guidance on the 'Safety assessment of botanicals and botanical preparations' (EFSA Scientific Committee, 2009) and the 'Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles' (EFSA Scientific Committee, 2021) have been followed by the FAF Panel for evaluating the present application.

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 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'.

To estimate the exposure to the food additive, nomenclature from the FoodEx2 classification system (EFSA, 2015) used in the Comprehensive Database was linked to the food categorisation system of Annex II to Regulation (EC) No 1333/2008, part D.

Uncertainties in the exposure assessment were identified and discussed (Section 3.3.2).

3 | ASSESSMENT

3.1 | Technical data

3.1.1 | Identity of the proposed food additive

The proposed food additive, named as jagua (genipin-glycine) blue is manufactured by water extraction of the ground pulp of the peeled unripe fruits of *Genipa americana* L., followed by filtration of the extract. The functional component, i.e. the blue polymer, is formed through a reaction between genipin, an iridoid compound naturally present in the extract and added glycine (authorised food additive E 640). The final product also consists of carbohydrates and proteins and is commercialised as a deep blue powder, containing a carrier (i.e. modified starch) (Documentation provided to EFSA Nos 1, 5).

The plant *G. americana* belongs to the Rubiaceae family and can be found, according to the applicant, in tropical areas and several regions of the subtropical areas of Latin America.

Scientific name of the plant: *Genipa americana* L. (1759).

Botanical synonyms: *Genipa americana L. var. Caruto (kunth) K.Schum, Genipa caruto Kunth*.

[4] https://www.ecfr.gov/current/title-21/chapter-I/subchapter-A/part-73/subpart-A/section-73.225.

[5] https://normas.mercosur.int/simfiles/normativas/105985_RES_003-2025_ES_Modificacion%20Res.%20GMC%2009-06%20y%2063-18.pdf.

Common names: Jagua, jagua blue, juito, huito, huito blue and genipa (Spanish areas of Latin America), genipap and genipa (English), bois de fer (French) and genipapo (Portuguese) (Documentation provided to EFSA No. 1).

The Panel noted that *G. americana* is included in the EFSA Compendium of Botanicals,[6] which lists botanicals that are reported to contain naturally occurring substances of possible concern for human health when present in food. In this case, coumarins, 2-propylfuran, furfural, phytic acid, proanthocyanidins (condensed tannins) and saponins are reported to occur in unspecified parts of the fruit, identified in alcoholic and organic extracts. Considering the manufacturing process of the proposed food additive (e.g. use of unripe fruits, water extraction, thermal treatment up to 70°C), and the fact that such substances are present in other food sources normally consumed, such as fruit, seeds and cereals, the Panel considered that, if present in the proposed food additive, they are not expected to occur at an amount that would raise a safety concern.

According to the applicant, the fruit is not usually consumed as such, but it is a popular material for the production of beverages in South America. Specifically, the pulp of the mature fruits is cooked with sugar to produce a syrup used for the preparation of various beverages and desserts (Documentation provided to EFSA No. 1).

In the unripe jagua fruit, different iridoids have been reported, such as genipin and geniposide (Bentes & Mercadante, 2014). According to the information provided by the applicant, only genipin is detected in the proposed food additive (Documentation provided to EFSA No. 4).

The unripe fruit is a source of genipin. Genipin is extracted from the fruit and reacts with added glycine to form the blue-coloured compound (blue polymer). This colour formation also occurs to a limited extent naturally in the unripe fruit and can be observed as dark blue veins on the fruit, when it is cut open. In contrast, the ripe fruit is not a good source of genipin, meaning that the blue colour cannot be observed any longer (Documentation provided to EFSA No. 1).

3.1.1.1 | *Blue colouring components*

The main source of the blue colour in the proposed food additive is a blue polymeric component. A detailed description of the identification of the main and minor blue colouring components of jagua (genipin-glycine) blue was provided by the applicant (Documentation provided to EFSA No. 1). The formation of the blue polymeric component is described by the applicant as consisting of three steps: monomer formation, dimer formation and ultimately, blue polymer formation.

3.1.1.1.1 | *Monomer formation*

According to the information provided by the applicant, the content of genipin in the water extract from the unripe jagua fruits is analysed and a stoichiometric quantity of glycine is added for the colour formation. The reaction with glycine produces an intermediate product, referred to by the applicant as 'monomer' (Figure 1) (Documentation provided to EFSA No. 1).

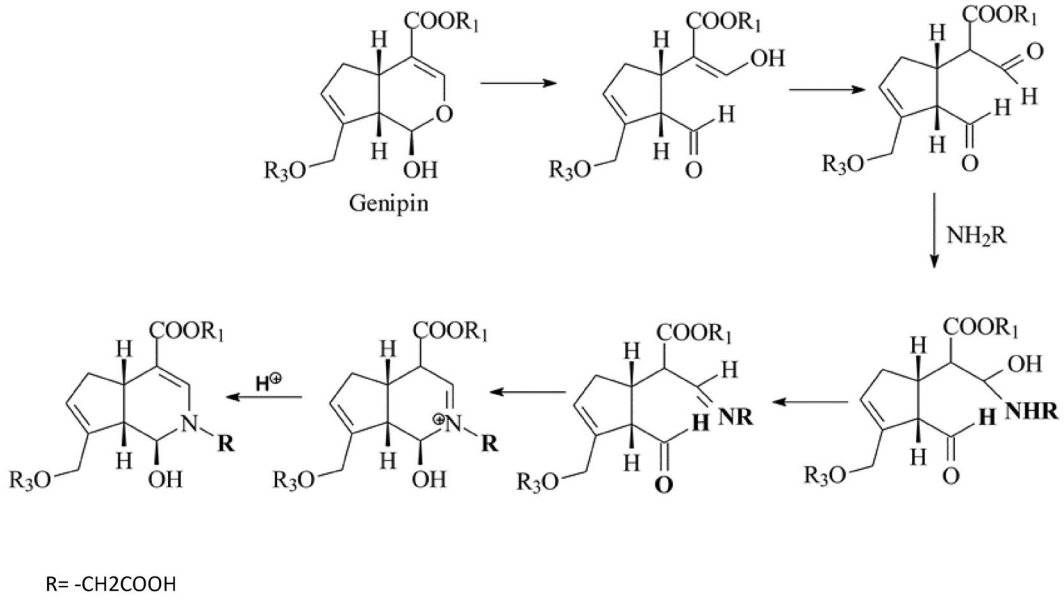


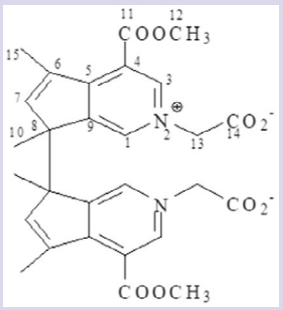
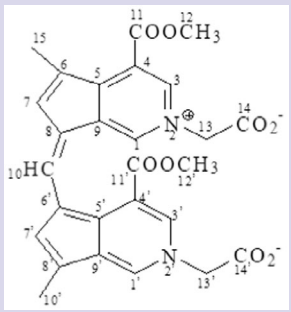
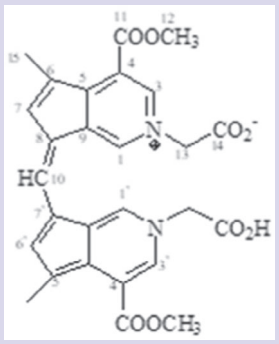
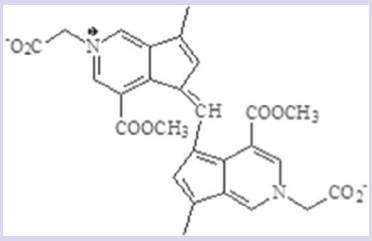
FIGURE 1 Mechanism for the reaction between genipin and glycine proposed by the applicant (Documentation provided to EFSA No. 1).

[6] https://www.efsa.europa.eu/en/data/compendium-botanicals.

3.1.1.1.2 | *Dimer formation*.

The monomer reacts further to form three different dimers, referred to by the applicant as 'Compounds 1, 2 and 3' (Table 1). These dimers react further to produce the intended blue polymer, which is the main colouring component, but this polymerisation is not complete and so the dimers are present as minor (subsidiary) colouring components in the proposed food additive. In the case of Compound 3, two possible structures (3A and 3B) are proposed by the applicant (Table 1). According to the applicant, structure 3A is more feasible since structure 3B has steric hindrance between the methyl ester groups of the two monomeric units, which could limit the formation and stability of the dimer structure 3B (Documentation provided to EFSA No. 1).

TABLE 1 Minor colouring components (dimers) of jagua (genipin-glycine) blue according to the applicant (Documentation provided to EFSA No. 1).

Compound	Compound 1	Compound 2	Compound 3
Formula	$C_{28}H_{28}N_2O_8$	$C_{27}H_{24}N_2O_8$	$C_{27}H_{24}N_2O_8$
Molecular weight	520	504	504
Chemical Name	7,7′-Bi-7*H*-cyclopenta[*c*]pyridinium, 2,2′-bis(carboxymethyl)-4,4′-(methoxycarbonyl)-5,5′,7,7′-tetramethyl-, bis(inner salt)	5H-Cyclopenta[*c*]pyridinium,2-(carboxymethyl)-5-[[2-(carboxymethyl)-4-(methoxycarbonyl)-5-methyl-2H-cyclopenta[*c*]pyridin-7-yl] methylene]-4-(methoxycarbonyl)-7-methyl-,inner salt	7*H*-Cyclopenta[*c*]pyridinium, 2-(carboxymethyl)-7-[[2-(carboxymethyl)-4-(methoxycarbonyl)-5-methyl-2H-cyclopenta[*c*]pyridine-7-yl] methylene]-4-(methoxycarbonyl)-5-methyl-, inner salt
CAS No.	1313734-13-2	104359-67-3	1313734-14-3
Melting point	> 300°C	> 300°C	> 300°C
Colour	Blue	Deep blue	Deep blue
Odour	Odourless	Odourless	Odourless
Structure			 3A structure  3B structure

The Panel noted that different representations of chemical structures are registered for the CAS numbers reported in Table 1.

3.1.1.1.3 | *Polymer formation*

The polymer (referred to by the applicant as 'Compound 4') (Figure 2) can be formed with all possible combinations of the three different dimers (Table 1). It is proposed by the applicant that two units of the polymer are attached through an ionic bond established between the cationic nitrogen and a carboxylate group. In addition, hydrogen bonding may occur between the carboxylic acid and methyl ester functional groups (Documentation provided to EFSA No. 1).

According to the applicant, the blue polymer has been identified and quantified with infra-red spectroscopy (IR), high-performance liquid chromatography–mass spectrometry (HPLC–MS) and high-performance liquid chromatography–diode array detection (HPLC–DAD) (at 590 nm), methods developed in-house (Documentation provided to EFSA No. 1).

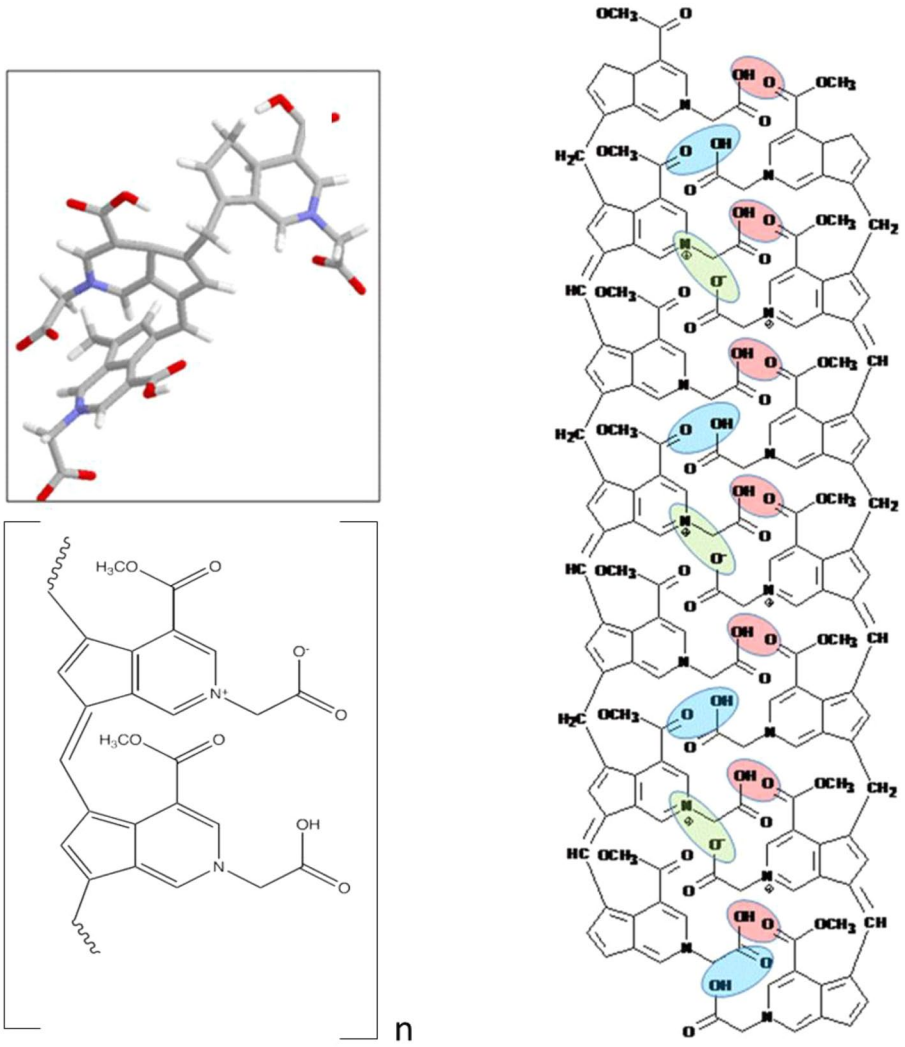


FIGURE 2 Illustrative chemical structure proposed for Compound 4 'the Polymer' with the chemical formula $(C_{27}H_{25}O_8N_2)_{10-12}$ according to the applicant (Documentation provided to EFSA No. 1).

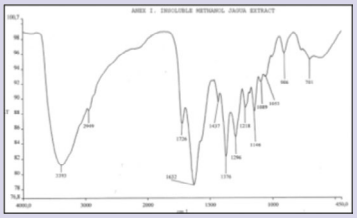
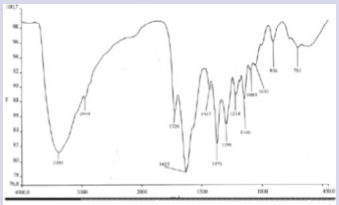
3.1.2 | Proposed specifications

The applicant provided specifications for the proposed food additive, i.e. jagua (genipin-glycine) blue (Table 2) (Documentation provided to EFSA No. 4, 7).

TABLE 2 Proposed specifications of jagua (genipin-glycine) blue as provided by the applicant and by JECFA 2020.

	Proposed by the applicant	JECFA (2020)
Synonyms	Jagua blue	Jagua blue
Definition	The colour additive Jagua (Genipin-Glycine) Blue is a deep blue powder prepared from the extract of unripe Jagua (*Genipa americana*) fruits, using water as an extraction solvent. The colour additive is the result of reaction between genipin (iridoid present in the fruit) and glycine. This reaction occurs naturally in the unripe fruits after being cut producing a mixture of blue compounds Jagua (Genipin-Glycine) Blue has a colour attributed mainly to a blue polymer that is formed by the reaction between genipin (methyl(1R,4aS,7aS)-1-hydroxy-7-(hydroxymethyl)-1,4a,5,7a-tetrahydrocyclopenta[c]pyran-4-carboxylate) and glycine resulting in the combination of alternating dimeric moieties linked by a methylene bridge	Jagua (Genipin-Glycine) Blue has a colour attributed mainly to a blue polymer that is formed by the reaction between genipin (methyl (1R,4aS,7aS)-1-hydroxy-7-(hydroxymethyl)-1,4a,5,7a-tetrahydrocyclopenta[c]pyran-4-carboxylate) and glycine resulting in the combination of alternating dimeric moieties linked by a methylene bridge Jagua (Genipin-Glycine) Blue is produced by a two-step process. In the first step the unripe fruit of *Genipa americana* is peeled, ground to pulp, pressed for the juice and extracted with water. The extracted juice is filtered and checked for genipin content. In the next step the jagua extract is treated with a stoichiometric amount of glycine and heated at 70° until all genipin is reacted. The resulting liquid is centrifuged and concentrated. The powder product in commerce is obtained after concentrating the Jagua (Genipin-Glycine) Blue to 20°Brix and mixing with a food-grade carrier such as maltodextrin or modified food starches, spray drying or using other drying technologies and sieving

TABLE 2 (Continued)

	Proposed by the applicant	JECFA (2020)
CAS Number	1314879-21-4	1314879-21-4 (Blue Polymer)
Chemical names	Glycine, reaction products with 2-(carboxymethyl)-5-([2-(carboxymethyl)-4- (methoxycarbonyl)-5-methyl-2H-cyclopenta[c]pyridin-7-yl]methylene]-4 methoxycarbonyl)-7-methyl-5H-cyclopenta[c] pyridinium inner salt homopolymer and methyl (1R, 4aS, 7aS)-1–4a,5,7a-tetrahydro-1-hydroxy-7-(hydroxymethyl) cyclopenta[c] pyran-4-carboxylate	–
Chemical formula	$(C_{27}H_{25}O_8N_2)_{10–12}$	$(C_{27}H_{25}O_8N_2)n$ (n is typically 10–12)
Molecular/atomic weight/ weight average molecular weight	Approximately 6000 Daltons	Approximately 6000 Da (number average molecular weight, approximately a lognormal distribution between 4500 and 9500 Da)
Assay	Not less than 20% and not more than 40% of polymer by HPLC	Not less than 30% and not more than 40% of polymer by HPLC
Description	Dark blue powder	Blue to black powder; odourless
Appearance of a solution	Bright blue	–
Identification		
Spectrophotometry	The UV–Visible absorption spectrum of a sample dissolved in water shows absorption maximum between 590 and 594 nm	The UV–Visible absorption spectrum of a sample dissolved in water shows absorption maximum between 590 and 594 nm
IR of Compound 4 'the Polymer'		 IR Spectrum of the blue polymer in Jagua (Genipin-Glycine) Blue shows characteristic bands at: 3393, 2949, 1726, 1630 and 1540 cm^{-1}.
Density/specific gravity	0.4–0.6 g/mL (25°C)	–
pH	4.0–5.5 (1% aqueous solution)	–
Melting range	180–195°C	–
Solubility	It is highly soluble in water. It is insoluble in non-polar solvents	Freely soluble in water. Practically insoluble to insoluble in hexane and ethanol
Purity		
Colour Value (E10%) at 590 nm	140–280	–
Total colouring matters %	–	35%–48%
Blue polymer	20%–40%	–
Minor colouring compounds	< 0.4%	–
Genipin content	< 10 ppm (< 0.001%)	Passes test (< 0.01%)
Glycine content	< 200 ppm (< 0.02%)	–
Loss on drying	< 8% at 105°C	Not more than 6% (at 105°, to constant weight)
Loss on ignition	< 10% at 600°C	–
Water insoluble matter	–	Not more than 0.2%
Ether-extractable matter	–	Not more than 0.2%
Water content	< 8% at 105°C	–
Ash	Not more than 10% (600°C)	–
Carbohydrates	< 85%	–
Total protein	< 10%	–
Total fat	< 10%	–
Modified starch	20%–30%	–
Mercury	Not more than 1 mg/kg	–

(Continues)

TABLE 2 (Continued)

	Proposed by the applicant	JECFA (2020)
Cadmium	Not more than 1 mg/kg	Not more than 1 mg/kg
Arsenic	Not more than 1 mg/kg	Not more than 1 mg/kg
Lead	Not more than 1 mg/kg	Not more than 2 mg/kg
Microbiological criteria		
Salmonella spp.	Negative (Absent in 25 g)	–
Escherichia Coli	Negative	Absent in 25 g of sample
Coagulase positive *Staphylococcus aureus*	Negative (Absent in 1 g)	Absent in 1 g of sample
Yeasts and moulds	< 100 CFU/g	Not more than 10 CFU/g
Total coliforms	< 10 CFU/g	Not more than 10 CFU/g
Total bacterial count (aerobic viable count)	< 1000 CFU/g	Not more than 1000 CFU/g

The manufacturing process as proposed by the applicant for inclusion in the specifications is rather generic. The Panel would propose a more detailed description of the manufacturing process of the proposed food additive (see Section 3.1.3) in the Definition. Specifically, it should be noted that the content of genipin in the unripe fruits is calculated, and stoichiometric amounts of glycine are added for the blue polymer to be formed. A carrier is also added to prepare the final preparation in powder form. In addition, the Panel noted that the proposal to include 'This reaction occurs naturally in the unripe fruits after being cut producing a mixture of blue compounds' in the Definition is not relevant to the manufacturing of the proposed food additive and is not necessary.

The Panel noted that the CAS Number proposed in the specifications of the applicant (1314879-21-4) refers to the blue polymer. The chemical names proposed refer to the reactants (genipin and glycine) and not the blue polymer.

Analytical results on 13 independently produced batches of the jagua (genipin-glycine) blue have been provided to demonstrate that each batch complies with the proposed specifications, as described below (Documentation provided to EFSA Nos 1–6).

Polymer and minor colouring compounds

Five different batches of jagua (genipin-glycine) blue were analysed for the presence of the blue polymer and minor colouring compounds by HPLC–DAD (Documentation provided to EFSA No. 1). The polymer's (Compound 4) concentration ranged from 31.72% to 34.92%, while the minor colouring compounds' concentration (sum of Compounds 1, 2 and 3) ranged from 0.14% to 0.20%. Based on the analytical data, the Panel noted that the proposed food additive is manufactured in compliance with the proposed specifications for those parameters.

Colour: E10%

The colour of the proposed food additive was expressed as the value E10% (590 nm) in five analysed batches and was reported between 243% and 273% (Documentation provided to EFSA No. 1), in accordance with the proposed specification range. However, the Panel noted that the proposed specification range of 140%–280% is considered to be wide, compared to the analytical data provided.

Genipin, geniposide and glycine

The same five batches of jagua (genipin-glycine) blue were analysed for the presence of genipin and glycine (Documentation provided to EFSA No. 1). Genipin was found to be below the limit of quantification (LOQ) (0.001% = 10 ppm) of the method in all five batches, and glycine was reported below the limit of detection (LOD) (0.0015% = 15 ppm) of the method in all five batches. In addition, the applicant provided analytical data for geniposide on 15 independently produced batches of the proposed food additive, which was reported to be below the LOD (0.00061% = 6.1 ppm) of the method in all analysed batches (Documentation provided to EFSA No. 6).

Composition

The applicant provided analytical data for total carbohydrates, sugars, modified starch, total fat, total protein, ash and moisture on the same five independently produced batches of the proposed food additive (Documentation provided to EFSA No. 1). Total carbohydrates (calculated by difference) ranged from 82.51% to 83.55%, fructose from 0.97% to 1.91%, glucose from 13.51% to 18.95%, sucrose from 7.51% to 9.56% and modified starch (the carrier, calculated as the total carbohydrates

minus soluble sugars) from 19.97% to 25.82%. In the case of total fat, the concentration ranged from 0.21% to 1.99%, of total protein from 5.32% to 9.82%, of ash from 3.60% to 7.12% and of moisture from 3.53% to 4.80%.

The analytical data show that the proposed limits for these components are met. However, the Panel noted that the actually measured concentrations for total fat (0.31%–1.99%) are substantially lower than the proposed specification limit of 10% for this parameter. In addition, the Panel noted that total carbohydrates (TC) were calculated by the applicant by difference (TC% = 100% – protein% – fat% – moisture% – ash%), without considering the functional component (blue polymer = 20% to 40%, according to the specifications), the minor blue colouring components and other components. Modified starch (MS) (which corresponds to maltodextrins used as carrier) was also calculated by difference (MS% = total carbohydrates% – fructose% – glucose% – sucrose%).

Pesticide residues

Pesticide residues (namely organochlorines, organophosphorus, pyrethroid and other pesticides) were routinely tested. The applicant provided analysis by gas chromatography tandem mass spectrometry (GC–MS/MS) and liquid chromatography tandem mass spectrometry (LC–MS/MS) of five batches of jagua (genipin-glycine) blue and no pesticides residues were detected (Documentation provided to EFSA No. 1). The applicant indicated that jagua fruits are mainly harvested from wild trees of *G. americana* found in the Colombian rainforest. However, in addition to wild harvesting, the applicant highlighted that cultivation programs with jagua trees have been established in the last years in areas where no pesticides are used (Documentation provided to EFSA No. 1). Despite the botanical origin of the proposed food additive, the Panel considered that no limits for pesticides in the proposed specifications of jagua (genipin-glycine) blue are needed, as long as it is assured that the fruits are collected from plants grown in low-risk areas for incidental contamination by pesticides or from plants that are not commercially cultivated. Should the proposed food additive be prepared in full or in part from cultivated *G. americana*, limits for pesticides should be considered for inclusion in the EU specifications.

Mycotoxins

Due to the botanical origin of the proposed food additive, the applicant provided analytical data for mycotoxins using LC–MS/MS on six independently produced batches of the proposed food additive (Documentation provided to EFSA No. 2). Based on the data, the applicant indicated that all values of mycotoxins (aflatoxins B1, B2, G1, G2, cytochalasin E, fumonisin B1 + B2, nivalenol, ochratoxin A, T-2 + HT-2 toxin, deoxynivalenol, 3 + 15 acetyl-deoxynivalenol, zearalenone) reported for the six samples of jagua (genipin-glycine) blue were below the reported LODs (ranging from 1 to 50 μg/kg). The Panel considered that there is no concern with respect to a potential contamination by mycotoxins in the proposed food additive and no need to introduce limits for mycotoxins in the EU specifications.

Toxic elements

Regarding toxic elements, results for the analysis of arsenic (As), lead (Pb), cadmium (Cd) and mercury (Hg) in five batches were reported (< 0.05 mg/kg for As in all batches, up to 0.240 mg/kg for Pb, < 0.01 mg/kg for Cd in all batches and < 0.01 mg/kg for Hg in all batches). Toxic elements were analysed with inductively coupled plasma mass spectrometry (ICP–MS) (Documentation provided to EFSA No. 1). Based on the analytical data, the Panel noted that the reported analytical data are substantially below the specification limits proposed for those toxic elements (< 1 mg/kg). Information on the anticipated exposure to toxic elements is reported in Section 3.3.3.

Microbiological criteria

Ten different batches were analysed for the presence of possible microbiological criteria (Documentation provided to EFSA No. 1, 3). Individual types of microorganisms, including *E. coli*, Enterobacteriaceae, *L. monocytogenes, Salmonella* spp. and *S. aureus*, were consistently not detectable in all tested batches. Total coliforms, aerobic mesophiles microorganism and spore counts, *Clostridium* sulfite reducing microorganism and spore count as well as yeasts and moulds count resulted to be 10 or below 10 CFU/g in all analysed batches. The Panel noted that for yeasts and moulds, a specification limit of below 100 CFU/g is proposed by the applicant, although based on the analytical data lower levels could be considered. However, the Panel considered that the data provided support the proposed specifications.

Particle size distribution

The applicant provided information on particle size distribution (PSD) of five batches of jagua (genipin-glycine) blue determined by laser diffraction (LD) (Documentation provided to EFSA No. 1). The method was described, and the descriptive analysis was provided. The Panel noted that LD analysis is not considered a proper method to investigate the presence of nanosized particles as it does not provide information on the size of the constituent particles as required by the EFSA Guidance-TR (EFSA Scientific Committee, 2021) and is prone to errors for polydisperse materials.

Following a request from EFSA for additional information, the applicant provided results from scanning electron microscopy (SEM) analysis coupled with energy dispersive X-ray (EDX) microprobe on five batches of jagua (genipin-glycine)

blue (4 of them being the same batches analysed initially by LD) (Documentation provided to EFSA No. 3). The particle size analysis method was described in detail. The applicant reported that the measured particles were of non-geometrical or spherical morphology and that the particle size was determined by measuring minimum Feret diameter of the constituent particles as requested in the EFSA Guidance-TR (by using an image analysis software). For each batch of jagua (genipin-glycine) blue, over 300 representative particles were analysed, and number-based size distributions and descriptive statistics were presented. The results of analysis provided showed that, for all batches analysed, the percentage of particles with one dimension smaller than 250 nm ranged from 73% to 98%, the median size of the particles ranged from 76 to 116 nm and 10% of all particles in all batches ranged from 40 to 48 nm.

The applicant also provided results of the volume specific surface area (VSSA) analysis (Documentation provided to EFSA No. 3). The Panel noted that the Brunauer–Emmett–Teller (BET) is not considered a proper method to investigate the presence of the fraction of small particles including nanoparticles as it cannot provide information on the constituent particles which is a requirement of the EFSA Guidance-TR (EFSA Scientific Committee, 2021).

The Panel considered that, based on the data provided, the presence of small particles including nanoparticles in jagua (genipin-glycine) blue as produced is confirmed. However, the Panel could not assess if the identified particles belong to the blue polymer only or to the other components (sugars, carbohydrates, proteins, small amounts of ashes and modified starches) of the proposed food additive, which are not of interest for this risk assessment.

Solubility

The applicant provided several water solubility tests in response to EFSA's requests. However, all the information provided (i.e. USP-NF-2021 method, shake flask method according to OECD TG 105 (OECD, 1995) without ultrafiltration step 3–10 kDa and with total carbon content (TOC) analysis) were not in line with EFSA Guidance-TR (EFSA Scientific Committee, 2021) as no ultrafiltration step was applied or no substance specific analysis was used to determine the concentration of the blue polymer (Documentation provided to EFSA No. 2, 3). The Panel was of the view that the above information provided on solubility does not inform on the solubility of this proposed food additive.

Considering the particle size distribution data described above, the Panel requested water solubility data only of the blue polymer, as it has been identified as the component of potential toxicological concern and the particulate matter originating from the fruit itself or the carrier is not of interest for this risk assessment.

In response to EFSA's request, the applicant reported that the blue polymer, without the carrier, is not available in sufficient amounts for testing. Consequently, the applicant performed another water solubility test of the proposed food additive following OECD TG 105, using a 10 kDa ultrafiltration membrane. The concentration of the blue polymer was determined using HPLC methodology developed and validated by the testing laboratory specifically for quantifying only the blue polymer content in the solution of jagua (genipin-glycine) blue (Document provided to EFSA No. 5).

The applicant stated that preliminary testing indicated a solubility for the blue polymer in the range of 100–200 g/L. However, difficulties were encountered during testing using the 'Flask Method' and ultrafiltration, due to the high viscosity of the solutions (750 g/L and 285 g/L). As a result, a water solubility value of approximately 6 g/L was determined for the blue polymer. The applicant also stated that further tests showed a significant amount of material (up to two-thirds) was lost during filtration. Therefore, the applicant proposed that the reported value of 6 g/L is likely an underestimation, with the true value likely closer to the preliminary solubility range of 100–200 g/L. The applicant further suggested that the OECD TG 105 and ultrafiltration method may not be suitable for determining the solubility of this substance (Document provided to EFSA No. 5).

The Panel noted that the conducted study does not support the applicant's claim that the solubility of the blue polymer is in the range of 100–200 g/L. Based on the data provided, the Panel concluded that the solubility of the blue polymer could only be considered approximately 6 g/L, which however may be underestimated.

3.1.3 | Manufacturing process

Jagua (genipin-glycine) blue is manufactured by water extraction of the ground pulp of the peeled unripe fruits of *G. americana*, followed by filtration of the extract. The genipin content of the extract is determined and a stoichiometric amount of glycine is added. The applicant mentioned that glycine used in the manufacturing process meets the specifications of the authorised food additive E 640. According to the applicant, the mixture is heated to 70°C for at least 2 h, until the blue colour is completely formed and the content of residual genipin and glycine is below 10 and 200 ppm, respectively, as reported in the proposed specifications. The liquid is centrifuged, concentrated and dried. Specifically, the final powder form of the proposed food additive is obtained by concentrating the mixture to 20°Brix and mixing with a carrier, such as maltodextrin or modified starch, spray dried, ground, sieved, packaged and stored (Documentation provided to EFSA No. 1–3, 7).

3.1.4 | Stability, reaction and fate in foods of the proposed food additive

The stability of jagua (genipin-glycine) blue powder was tested in five batches of the proposed food additive when packed in high barrier plastic bags and held at room temperature (18–34°C) and relative humidity (RH) (30%–85%) for up to 48

months. Analysis of the samples at intervals was by UV–VIS spectroscopy, HPLC (SEC)–DAD and LC–MS. A powder sample was also tested for stability at 176°C for 20 min (Documentation provided to EFSA No. 1). There was no detectable loss in colour and no new HPLC peaks formed after 48 months storage at room temperature. After the 176°C/20 min treatment, the colour intensity diminished by 12% and this was accompanied by the formation of higher molecular weight polymeric material. No new lower molecular weight peaks were seen in the analysis. The applicant attributed the changes to a progression of the polymerisation process at the high temperature.

A series of normal and accelerated stability studies were conducted on a wide range of foods that had added jagua (genipin-glycine) blue. The foods and the test conditions comprised: flavoured milk (1.7% fat, pH 6.6, 28 days at 4°C), yogurt (2% fat, pH 4, 42 days at 4°C), ice cream (12% fat, pH 6.6, 6 months at −18°C), cream cheese (27% fat, pH 4.6, 22 days at 4°C), extruded cereal product (maize/rice, 82 days at 25, 35 and 45°C, and including a 177°C/3 min extrusion), potato chips (surface coated, 85 days at 25, 35 and 45°C), jelly powder (gelatin, 83 days at 25, 35 and 45°C), ready-to-eat jelly (set water-gelatin, 43 days at 25°C), gummies (sugar/gelatin, 92 days at 4, 25 and 35°C), hard sugar candy (85 days at 20 and 25°C), chocolate dragées (84 days at 4, 25 and 35°C), chewing gum (63 days at 20 and 25°C), nutritional beverage powder (84 days at 25, 35 and 45°C) and soy milk (1.5% fat, pH 6, 29 days at 4 and 25°C). The parameters monitored over the time course were the blue polymer content (%), colour loss rate, the time calculated to give rise to a 10% loss of the colour and the possible formation of degradation products (Documentation provided to EFSA No. 1). The Panel noted that stability of the proposed food additive in the food products tested was demonstrated. Further, from the HPLC chromatograms provided, there was no evidence of formation of degradation products from the blue polymer under the conditions studied.

The Panel considered jagua (genipin-glycine) blue to be stable as a powder and in food matrices under the evaluated conditions.

3.1.5 | Methods of analysis in food

According to the applicant, an HPLC method with DAD has been established for the routine quantification of the blue polymer (functional component of jagua (genipin-glycine) blue) in different food matrices (dairy products, snack and cereal products, confectionery and beverages) (Documentation provided to EFSA No. 1).

3.2 | Proposed uses and use levels

Through the current application, authorisation of jagua (genipin-glycine) blue as a food additive is sought with regards to the food categories listed in Table 3.

The Panel noted that the applicant submitted proposed maximum and typical use levels of the food additive jagua (genipin-glycine) blue (in mg/kg) for 16 food categories (FCs) according to Annex II, part D of Regulation (EC) No. 1333/2008. Proposed use levels were also expressed as blue polymer (functional component) considering a 40% content in the proposed additive jagua (genipin-glycine) blue.

TABLE 3 Proposed uses and use levels of jagua (genipin-glycine) blue expressed in jagua (genipin-glycine) blue and in the blue polymer (in mg/kg) (Documentation provided to EFSA No. 5, 8).

Food category number	Food category name	Restrictions/exceptions	Proposed maximum use levels (mg/kg)	Proposed typical use levels (mg/kg)	Proposed maximum use levels expressed as blue polymer considering 40% content (mg/kg)	Proposed typical use levels expressed as blue polymer considering 40% content (mg/kg)
01.4	Flavoured fermented milk products, including heat-treated products	Yogurt, regular and Greek	300	200	120	80
01.7.5	Processed cheese	Cream cheese-based spread, flavoured	200	110	80	44
01.8	Dairy analogues, including beverage whiteners	Milk substitutes	400	250	160	100
		Yogurt, dairy alternative	500	260	200	104
03	Edible ices	Ice cream and frozen dairy and dairy alternative desserts	1000	700	400	280
		Gelatins, ices, sorbets	300	160	120	64
04.2.4.1	Fruit and vegetable preparations excluding compote	Fruit toppings & fillings	300	160	120	64
04.2.4.2	Compote, excluding products covered by category 16				120	64
04.2.5.2	Jam, jellies and marmalades and sweetened chestnut puree as defined by Directive 2001/113/EC	Jam, jellies and marmalade	300	160	120	64
04.2.5.3	Other similar fruit or vegetable spreads					
05.2	Other confectionery including breath refreshening microsweets	Confectionery containing chocolate	2000	500	800	200
		Confectionery not containing chocolate	2000	500	800	200
05.3	Chewing gum	Chewing gum	2000	1500	800	600
05.4	Decorations, coatings and fillings, except fruit-based fillings	Icing & frosting	300	160	120	64
		Syrups/toppings for beverages, desserts and breakfast syrups	300	160	120	64
06.3	Breakfast cereals	RTE cereal, multi-coloured	5000	2900	2000	1160
13.2	Dietary foods for special medical purposes defined in Directive 1999/21/EC	Nutritional beverages (RTD and powders)	200	160	80	64
14.1.4	Flavoured drinks	Flavoured milk	400	210	160	84
		Milk shakes	400	210	160	84
		Other dairy drinks	400	210	160	84
		Smoothies	400	160	160	64
15.1	Potato-, cereal-, flour- or starch-based snacks	Potato chips, flavoured	2000	1540	800	616
		Tortilla, corn, other chips	3000	1540	1200	616
16	Desserts	Pudding	300	210	120	84

3.3 | Exposure assessment

According to the current 'Guidance for submission for food additive evaluation' (EFSA ANS Panel, 2012), the applicant provided estimates of exposure to the proposed food additive using the FAIM template based on both proposed maximum/typical use levels (Documentation provided to EFSA No. 1, 2). The applicant also provided a more refined exposure estimate based on DietEx template with proposed maximum/typical use levels detailed at FoodEx2 Level 5 (Documentation provided to EFSA No. 2).

The Panel considered that the exposure estimates resulting from the DietEx tool reflect better the proposed uses from the applicant (Table 3) as this tool allows a selection of the foods at the FoodEx2 level (and not at the level of the food categories as in FAIM). Thus, this tool provides more realistic estimates of exposure, compared to FAIM. Therefore, only results from this tool are shown below.

3.3.1 | Exposure data

Food consumption data used for exposure assessment

EFSA Comprehensive European Food Consumption Database

Food consumption data of infants, toddlers, children, adolescents, adults and the elderly in the Comprehensive Database were used for this exposure assessment. Food consumption data were available from 43 different dietary surveys carried out in 22 European countries.[7] The details of the population groups considered and the countries with food consumption surveys available are presented in Annex A, Table A1.

Food categories considered for the exposure assessment of jagua (genipin-glycine) blue

Using DietEx, the foods belonging to the food categories in which the use of jagua (genipin-glycine) blue is proposed were selected at the most detailed level possible (up to Level 5 of the FoodEx2 classification system) (EFSA, 2015). The restrictions/exceptions proposed by the applicant were considered for all proposed food categories in the exposure assessment performed with DietEx. However, the restriction 'Icing & frosting' in FC 05.4 'Decorations, coatings and fillings' could not be attributed to any food within DietEx and was therefore not considered in the exposure assessment. Therefore, all foods belonging to FC 05.4 were considered.

The applicant did not consider all foods within FC 05.2 'Other confectionery' in their exposure assessment using DietEx. The Panel noted that there is no restriction within this food category and therefore considered all foods belonging to FC 05.2.

3.3.2 | Exposure to jagua (genipin-glycine) blue from its proposed use as food additive

Estimate of exposure based on the DietEx Tool

The applicant provided an estimate of exposure to jagua (genipin-glycine) blue and its blue polymer based on the output obtained from DietEx at the proposed maximum/typical use levels submitted (Documentation provided to EFSA No. 2). The proposed maximum and typical use levels for the food additive and its blue polymer were provided as described in Table 3. For estimating the exposure to the blue polymer, the upper end of the colour content distribution range of 40% as described in the specifications (Table 2), was applied by the applicant.

For the reason mentioned above regarding the inclusion of all foods within FC 05.2 'Other confectionery', the Panel calculated the exposure to the food additive and its blue polymer using DietEx for both the proposed maximum and typical use level exposure assessment scenario. The summary of the results per population group is provided in Tables 4 and 5, respectively. Detailed results per population group and survey are presented in Annex A, Table A2.

[7]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 Annex A.

TABLE 4 Summary of dietary exposure to jagua (genipin-glycine) blue from its proposed maximum/typical use levels as a food additive in six population groups, estimated with DietEx (minimum–maximum across the dietary surveys in mg/kg bw per day), across all proposed food categories.

	Infants (0 week–11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly[a] (≥ 65 years)
Proposed maximum use level exposure assessment scenario						
Mean	0.5–4.7	2.5–**9.4**	2.4–6.8	1.1–4.1	0.6–2.1	0.2–1.5
95th percentile	2.9–23.1	10.7–**26.9**	9.1–20.4	3.7–11.3	2.5–6.2	1.2–6.0
Proposed typical use level exposure assessment scenario						
Mean	0.3–2.8	1.5–5.7	1.4–4	0.6–2.2	0.3–1.2	0.1–0.9
95th percentile	1.8–13.3	5.3–**15.8**	5.0–11.3	1.8–6.5	1.5–3.5	0.7–3.6

[a]DietEx provides exposure estimates for elderly and very elderly populations. To ease the reading, and for consistency with previous opinions related to food additives, exposure results are here reported as the range between both populations (i.e. the min being the minimum among both populations and max being the maximum among both populations).

TABLE 5 Summary of dietary exposure to the blue polymer in jagua (genipin-glycine) blue from its proposed maximum/typical use levels as a food additive in six population groups, estimated with DietEx (minimum–maximum across the dietary surveys in mg/kg bw per day), across all proposed food categories.

	Infants (0 week–11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly[a] (≥ 65 years)
Proposed maximum use level exposure assessment scenario[b]						
Mean	0.2–1.9	1.0–3.8	0.9–2.7	0.4–1.6	0.2–0.8	0.1–0.6
95th percentile	1.2–9.2	4.3–**10.8**	3.7–8.2	1.5–4.5	1.0–2.5	0.5–2.4
Proposed typical use level exposure assessment scenario[b]						
Mean	0.1–1.1	0.6–2.3	0.5–1.6	0.2–0.9	0.1–0.5	0.1–0.3
95th percentile	0.7–5.3	2.1–**6.3**	2.0–4.5	0.7–2.6	0.6–1.4	0.3–1.4

[a]DietEx provides exposure estimates for elderly and very elderly populations. To ease the reading, and for consistency with previous opinions related to food additives, exposure results are here reported as the range between both populations (i.e. the min being the minimum between among both populations and max being the maximum among both populations).

[b]Calculations based on the maximum concentration of 40% of the blue polymer in the food additive reported by the applicant in the specifications (Table 2).

Main foods contributing to exposure to jagua (genipin-glycine) blue or the blue polymer based on the DietEx tool

Using both the proposed maximum and typical use levels, the main foods contributing to the total mean exposure estimates for all population groups were 'Yogurt' (belonging to FC 01.4 'Flavoured fermented milk products, including heat-treated products') and 'Processed and mixed breakfast cereals' (belonging to FC 06.3 'Breakfast cereals'). For children, adolescents and the elderly, 'Ice cream, milk-based' (belong to FC 03 'Edible ices') was also an important contributor to the exposure.

Annex A, Table A3 indicates all the contributing foods by population groups.

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 have been considered and summarised in Table 6.

TABLE 6 Qualitative evaluation of influence of uncertainties on the dietary exposure estimate.

Sources of uncertainties	Direction[a]
Consumption data	
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/–
Methodology used to estimate high percentiles (95th) long-term (chronic) exposure based on data from food consumption surveys covering only a few days	+
Concentration data	
Correspondence of proposed use levels to the food items in the EFSA Comprehensive Database: uncertainties to which types of food the levels refer to	+/–
Uncertainty in possible national differences in use levels of food categories	+/–
Proposed maximum/typical use levels considered applicable to all foods within the proposed ones in the food category, whereas most probably not all foods will contain jagua (genipin-glycine) blue as a food additive	+
It was assumed the concentration of the blue polymer in jagua (genipin-glycine) blue is at the maximum of the colour content distribution range of 40%, while it can contain between 20 and 40% of blue polymer.	+

TABLE 6 (Continued)

Sources of uncertainties	Direction[a]
Methodology	
Proposed use level exposure assessment scenario:	
Exposure calculations based on the proposed typical use levels	+/–
Exposure calculations based on the proposed maximum use levels	+

[a]+, uncertainty with potential to cause overestimation of exposure; –, uncertainty with potential to cause underestimation of exposure.

Jagua (genipin-glycine) blue is requested to be authorised in 16 food categories. For all food categories considered, it was assumed that 100% of the foods within the proposed food categories (see Table 3), that also meet the restrictions/exception indicated by the applicant except for one food category (FC 05.4, see above), will contain jagua (genipin-glycine) blue at the proposed maximum/typical use levels. As it is not likely that this will be the case in practice, the Panel considered overall that the uncertainties identified resulted in an overestimation of the exposure at both the typical and the maximum proposed use levels.

3.3.3 | Anticipated exposure to toxic elements from proposed specifications

The applicant provided proposals for maximum limits for As (< 1.0 mg/kg), Pb (< 1.0 mg/kg), Cd (< 1.0 mg/kg) and Hg (< 1.0 mg/kg) for the specifications of the proposed food additive (see Table 1). The Panel noted that the occurrence data on toxic elements for five independently produced batches of the proposed food additive submitted by the applicant are much lower than the proposed specification limits, being < 0.05 mg/kg for As, 0.051–0.240 mg/kg for Pb, < 0.01 mg/kg for Cd and < 0.01 mg/kg for Hg.

The potential exposure to impurities from the use of the proposed food additive was calculated by assuming that they are present in the food additive up to a certain limit value and then by calculation pro-rata to the estimates of exposure to the food additive itself.

Considering that the blue polymer represents between 20% and 40% of the proposed food additive, no significant change in exposure to toxic elements as impurities is expected whether the levels are expressed as a percentage of the total food additive or as a percentage of the blue polymer, i.e. functional component. Therefore, the proposed limits and analytical data expressed in percentage of the total food additive were used for assessing the risk that would result if these toxic elements are present in the proposed food additive. The highest refined exposure levels using the DietEx tool for the mean and 95th percentile among the different population groups at the proposed maximum use level were considered, i.e. 9 and 27 mg/kg bw per day respectively, for toddlers (Table 4).

The potential levels of the toxic elements in the proposed food additive combined with the estimated exposure levels presented in Table 4, result in exposure estimates that can be compared with the following reference points (RP) or health based guidance value (HBGV) (Table 7) for the toxic elements. It is considered that any Hg or As in the proposed food additive corresponds to the element in the inorganic form rather than an organic form. Consequently, the HBGV for inorganic mercury and the RP for inorganic arsenic were used for comparison (Table 7).

TABLE 7 Reference points/health-based guidance value for toxic elements potentially present in the proposed food additive.

Impurity/HBGV/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)
Inorganic mercury (iHg)/4 mg/kg bw per week (TWI)	The HBGV was set using kidney weight changes in male rats as the pivotal effect. Based on the $BMDL_{10}$ of 0.06 mg/kg bw per day, expressed as mercury and an uncertainty factor of 100 to account for inter and intra species differences, with conversion to a weekly basis and rounding to one significant figure, a TWI for inorganic mercury of 4 μg/kg bw per week, expressed as mercury was established EFSA CONTAM Panel (2012)
Cadmium (Cd)/2.5 mg/kg bw per week (TWI)	The derivation of the reference point is based on a meta-analysis to evaluate the dose–response relationship between selected urinary cadmium and urinary beta-2-microglobulin as the biomarker of tubular damage recognised as the most useful biomarker in relation to tubular effects. A group-based $BMDL_5$ of 4 μg Cd/g creatinine for humans was derived. A chemical specific adjustment factor of 3.9 was applied to account for human variability in urinary cadmium within each dose-subgroup in the analysis resulting in a reference point of 1.0 μg Cd per g creatinine. In order to remain below 1 μg Cd/g creatinine in urine in 95% of the population by age 50, the average daily dietary cadmium intake should not exceed 0.36 μg Cd/kg bw, corresponding to a weekly dietary intake of 2.5 μg Cd/kg bw EFSA CONTAM Panel (2009)

(Continues)

TABLE 7 (Continued)

Impurity/HBGV/RP	Basis/reference
Inorganic arsenic (iAs)/0.06 µg/kg bw per day (BMDL05)	The reference point is based on a benchmark dose lower confidence limit (BMDL05) of 0.06 µg/kg bw per day identified for skin cancer. The reference point is considered to cover lung cancer, bladder cancer, skin lesions, ischemic heart disease, chronic kidney disease, respiratory disease, spontaneous abortion, stillbirth, infant mortality and neurodevelopmental effects. A MOE of 1 would correspond to the exposure level that is associated with a 5% increase relative to the background incidence for skin cancer, based on the available data. A MOE of 1 raises a health concern. Because there are no precedents in EFSA for identification of a MOE of low concern, when using a BMDL derived from human cancer data the CONTAM Panel decided not to determine a value for a MOE of low concern EFSA CONTAM Panel (2024)

Abbreviations: BMDL01, benchmark dose (lower confidence limit); HBGV, health-based guidance value; MOE, margin of exposure; RP, reference point; TWI, tolerable weekly intake.

The risk assessment of the impurities helps to determine whether there could be a possible health concern if these impurities would be present at a certain level in the proposed food additive. The assessment is performed by calculating e.g. the MOE (margin of exposure) by dividing the RP (i.e. BMDL, Table 7) by the exposure estimate (Table 4).

The Panel assessed the risk that would result if these toxic elements were present in jagua (genipin-glycine) blue according to two concentration scenarios: (i) considering their presence at the proposed specification limits and (ii) considering the analytical data provided at the rounded up highest measured value (i.e. for lead) and, in the absence of any measured value, at the reporting limit (i.e. for arsenic, cadmium, mercury), modulated by the Panel by applying a factor of 10 to allow flexibility with respect to representativeness, homogeneity and differing analytical methods (Table 8).

TABLE 8 Risk assessment for toxic elements in jagua (genipin-glycine) blue.

Exposure to jagua (genipin-glycine) blue (mg/kg bw per day)	(i) Considering the presence of the toxic elements at the proposed specification limits			
	MOE for iAs at 1.0 mg/kg	**MOE for Pb at 1.0 mg/kg**	**% of the TWI for Cd at 1.0 mg/kg**	**% of the TWI for iHg at 1.0 mg/kg**
9[a]	6	53	3	2
27[b]	2	19	8	5
Exposure to jagua (genipin-glycine) blue (mg/kg bw per day)	**(ii) Considering the presence of the toxic elements at the rounded up highest measured value and, in the absence of any measured value, at the reporting limit, modulated by the Panel by applying a factor of 10**			
	MOE for iAs at 0.5 mg/kg	**MOE for Pb at 0.3 mg/kg**	**% of the TWI for Cd at 0.1 mg/kg**	**% of the TWI for iHg at 0.1 mg/kg**
9[a]	13	177	< 1	< 1
27[b]	5	66	< 1	< 1

Abbreviations: iAs, inorganic arsenic; bw, body weight; Cd, cadmium; iHg, inorganic mercury; MOE, margin of exposure; Pb, lead; TWI, tolerable weekly intake.

[a]Highest mean exposure among different population groups (proposed maximum use level exposure assessment scenario–toddlers, Table 4).

[b]Highest 95th percentile exposure among different population groups (proposed maximum use level exposure assessment scenario–toddlers, Table 4).

Considering the results of the exposure to the toxic elements Pb, Cd and iHg, the Panel noted that their presence in jagua (genipin-glycine) blue at both scenarios would not give rise to concern. In the case of iAs, the Panel noted that its presence at the proposed specification limit value of 1 mg/kg would lead to an MOE of 2.2.

The Panel recommends to lower the specification limits proposed by the applicant for all four toxic elements (Pb, Cd, iHg, iAs), taking into account (i) the results of the calculations performed by the Panel (the MOE for iAs is close to the MOE of 1 at which risk cannot be excluded, Table 8), (ii) the fact that the proposed food additive is not the only potential dietary source of toxic elements and that (iii) the maximum limits should be established based on actual levels in the commercial food additive.

The Panel considered that the choice of maximum limits for toxic elements in the specifications is in the remit of risk manager(s). The values used here were merely taken to support the risk assessment of these toxic elements as presented above.

3.4 | Biological and toxicological data

The Panel followed a conventional risk assessment as described in the 'Guidance for submission for food additive evaluation' (EFSA ANS Panel, 2012) since no concern was identified with regard to the potential presence of small particles, including nanoparticles of the blue polymer, at the proposed uses and use levels of the proposed food additive (see Section 4).

The Caco-2 cell permeability study, the repeated dose toxicity studies as well as the genotoxicity studies submitted in support of this application were performed with a test item referred to as '*jagua extract*'. Based on the information provided by the applicant, '*jagua extract*' is the same substance as jagua (genipin-glycine) blue. The certificates of analysis (CoAs) of the batches used in all the studies were provided by the applicant (Documentation provided to EFSA No. 1). The Panel noted that the test substance used in the evaluated studies is fully representative of the material of commerce of the new proposed food additive.

3.4.1 | Absorption, distribution, metabolism and excretion

In vitro studies

A Caco-2 cell permeability test with jagua extract was submitted by the applicant in order to examine the permeability of the proposed food additive across Caco-2 epithelial monolayers (Documentation provided to EFSA No. 1). This study was performed under non-GLP conditions. In incubations with the test item (200 mg/mL jagua extract, concentration of blue polymer 33.79%) for 2 h permeability through Caco-2 monolayers was measured both from apical to basolateral ($A \rightarrow B$, absorption) and from basolateral to apical ($B \rightarrow A$, efflux). In parallel incubations, the reference compounds ranitidine, talinolol and warfarin (10 µM each) served as low permeability, P-gp efflux and high permeability controls, respectively. The analysis of test item present in media samples from the receiver side (*A* or *B*) was conducted with a UV–Vis plate reader and compared to a standard curve, and by LC–MS/MS for the control substances (raw data not provided). Data were reported for the apparent permeability rate coefficient (P_{app}; 10^{-6} cm/s) and efflux ratio (P_{app} ($B \rightarrow A$))/P_{app} ($A \rightarrow B$).

Under the conditions of the in vitro absorption assay, jagua extract demonstrated low permeability as monitored by UV/Vis using the Caco-2 cell monolayer system. It exhibited a rate coefficient of 0.13×10^{-6} cm/s lower than 0.31×10^{-6} cm/s for ranitidine (the low permeability standard) and far below the 27×10^{-6} cm/s for warfarin (the high permeability standard). The efflux ratio of 5.6 for jagua extract suggested that it may even be actively transported out of the enterocytes; yet, a value for talinolol, the P-glycoprotein efflux control, was not reported in the study report. The Panel noted that the only concentration tested of jagua extract in the study was two orders or more of magnitude higher than the concentrations of the reference compounds (ranitidine, talinolol and warfarin) and this was considered a suboptimal test condition.

In vivo studies

In subsequent 28- and 90-day oral toxicity studies in dog (three animals/sex/group) (see also Section 3.4.3), plasma concentration of the blue polymer (test item jagua extract, concentration of blue polymer 33.79%; vehicle: sterile water) were measured. Blood samples were collected from all animals before treatment and at 0.5, 2, 4, 8 and 24 h after treatment at day 1 and 28 and day 1 and 91, respectively.

Blood samples (about 1 mL) were placed in heparinised tubes, pre-cooled and centrifuged to obtain plasma samples which were stored in a freezer at −80°C until analysis. The bioanalytical method with spectrophometric detection at 590 nm achieved a lower limit of quantification (LLOQ) of 1 mg/mL and an upper limit of quantification (ULOQ) of 2.5 mg/mL plasma. The blue polymer was not detected in plasma samples from animals dosed repeatedly with jagua extract either 250, 500 or 1000 mg/kg bw per day.

Both study reports mentioned that blue faeces were observed during the study in all animals treated with the test item, and further that most of the treated animals at all doses showed green-coloured urine and discolouration of some organs with a scale of increasing intensity related to the increasing dose.

The Panel noted that the method used in these studies to measure concentrations of the blue polymer in plasma had inadequate sensitivity by at least an order of magnitude.

According to the applicant, based on the tiered approach to toxicokinetic testing of the 'Guidance for submission for food additive evaluation' (EFSA ANS Panel, 2012), a low permeability of jagua extract was demonstrated in the Caco-2 permeability test and that this would deem necessary further testing. However, based on the absence of adverse treatment-related effects observed in the toxicity studies submitted within the dossier (see Sections 3.4.3 and 3.4.5), the applicant considered that no higher tiered toxicokinetic studies on the food additive are needed.

The Panel considered that based on the evidence from the toxicity studies (see Sections 3.4.3 and 3.4.5) some absorption of the proposed food additive has been demonstrated.

3.4.2 | Genotoxicity

3.4.2.1 | *Genotoxicity of jagua (genipin-glycine) blue*

The applicant firstly submitted data from two in vitro genotoxicity tests consisting of a bacterial reverse mutation assay and an in vitro mammalian cell gene mutation test. In addition, also an in vivo micronucleus test was provided. The studies were performed in compliance with GLP principles.

According to the EFSA strategy for genotoxicity testing, the bacterial reverse mutation assay and the in vitro micronucleus test are recommended as basic test battery (Tier 1) for hazard identification (EFSA ANS Panel, 2012; EFSA Scientific Committee, 2011). Upon request from EFSA, an in vitro micronucleus test (MN) in Chinese Hamster Ovary (CHO-K1) cells was submitted. The study was performed in compliance with GLP principles.

All studies presented below are also detailed in Appendix A.

In vitro studies

Bacterial reverse mutation test

The mutagenicity of jagua extract (concentration of blue polymer 33.62%[8]) was investigated in *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 102, with and without metabolic activation (rat liver S9 fraction), up to a maximum concentration of 5000 µg/plate, in compliance with OECD TG 471 (Documentation provided to EFSA No. 1).

The test item was dissolved in distilled water. Two independent experiments were performed, applying the plate incorporation method (experiment I) and the pre-incubation method (experiment II). Some toxicity was reported in experiment I only in the tester strain TA 1537 at concentrations of 2500 µg/plate and higher without S9 and in the experiment II in different strains at 100 µg/plate and higher without S9 and at 2500 µg/plate and higher with S9. This toxicity did not interfere with the performance of the test. No precipitation was observed.

No biologically relevant increases in revertant colony numbers were observed in any experimental condition. The positive control chemicals induced a distinct increase of revertant colonies, indicating the validity of the experiments.

The Panel considered this study reliable without restrictions and of high relevance.

In vitro gene mutation assay in mouse lymphoma L5178Y cell line

The test item jagua extract (concentration of blue polymer 33.62%[11]) was assessed for its potential to induce gene mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y, with and without metabolic activation (rat liver S9 fraction), in compliance with OECD TG 476 (OECD, 1997b) (Documentation provided to EFSA No. 1). Two treatment schedules were applied: a 4-h short-term exposure with and without metabolic activation; a 24-h long-term exposure, only without metabolic activation.

The test item was dissolved in culture medium RPMI plus horse serum (HS). The concentrations used in the main experiments were selected on the basis of cytotoxicity, calculated as relative total growth (RTG). In the short-term experiment the maximum concentration used was 5000 µg/mL with metabolic activation (RTG = 35.2%) and 4250 µg/mL without metabolic activation (RTG = 11.7%). In the long-term experiment the maximum concentration used was 5000 µg/mL with metabolic activation (RTG = 22.0%) and 3250 µg/mL without metabolic activation (RTG = 10.1%). No precipitation of the test item was noted.

No biologically relevant increase of mutants was found after treatment with the test item in any experimental condition. The global evaluation factor (GEF) was not exceeded by the induced mutant frequency at any concentration. No dose–response relationship was observed. Additionally, in both experiments colony sizing showed no clastogenic effects induced by the test item.

Ethyl methanesulfonate (EMS), methylmethanesulfonate (MMS) and benzo[a]pyrene (B[a]P) were used as positive controls. These chemicals showed distinct and biologically relevant effects in mutation frequency and MMS and B[a]P significantly increased the number of small colonies, thus proving the efficiency of the test system.

The Panel considered this study reliable without restrictions and of high relevance.

In vitro mammalian cell micronucleus test

The aneugenic and clastogenic impact of jagua extract (concentration of blue polymer 33.47%) was investigated in a micronucleus test in cultured CHO-K1 cells, with and without metabolic activation (rat liver S9 fraction) in compliance with OECD TG 487 (Documentation provided to EFSA No. 2).

The test item was dissolved in water. Based upon the cytotoxicity test, the concentrations selected for the micronucleus assay were 500, 1000, 2000 µg/mL. No precipitation was observed in this concentration range. Two treatment schedules were applied: 3 h treatment followed by a 20-h recovery time for the short-term experiment, with and without metabolic activation; 20 h continuous treatment for the long-term experiment, only without metabolic activation.

Cyclophosphamide monohydrate (CPA), mitomycin C (MYC) and vinblastine sulfate (VIN) were used as positive control chemicals. In particular, CPA and MYC were used as clastogenic controls in the presence and absence of S9, respectively, VIN was used as aneugenic control. All positive controls induced statistically significant increases in the proportion of cells with micronuclei. The maximum cytotoxicity observed was 22.28%, reported after the long-term treatment without S9.

The exposure of cells with jagua extract in the absence and presence of metabolic activation did not increase the frequency of micronuclei in CHO-K1 cells when compared with the untreated controls, therefore the test item did not show any potential to induce aneugenicity or clastogenicity under the experimental conditions used.

[8]As reported in the related certificate of analysis (CoA) (Documentation provided to EFSA No. 1).

The Panel considered this study reliable without restrictions and of high relevance.

In vivo studies

Mammalian erythrocyte micronucleus test

The potential of jagua extract (concentration of the blue polymer 33.62%[8]) to induce micronuclei in vivo was investigated in mouse bone marrow in compliance with OECD TG 474 (Documentation provided to EFSA No. 1). The test item (suspended in 0.9% NaCl) was administered intraperitoneally (i.p.) in 10 mL/kg bw. Under the experimental conditions reported, the test item did not induce structural and/or numerical chromosomal damage in the immature erythrocytes of the mouse. However, the study was performed using a route of administration (intraperitoneal) that is generally not relevant for the intended route of exposure to the food additive in humans (OECD TG 474). In particular, the intraperitoneal administration does not allow to reflect the metabolic fate of the tested material after oral exposure, therefore this study could not be considered by the Panel in the overall assessment.

The Panel considered this study not reliable and of low relevance.

Overall, jagua extract was negative in a bacterial reverse mutation assay performed in *S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537, in an in vitro gene mutation assay in mouse lymphoma L5178Y cell line and in an in vitro micronucleus test in CHO-K1 cells. Therefore, jagua extract does not raise a concern regarding genotoxicity.

3.4.2.2 | *Genotoxicity of minor colouring compounds of jagua (genipin-glycine) blue*

In view of the presence of low molecular weight components (see Section 3.1.1) in the proposed food additive jagua (genipin-glycine) blue, an in silico analysis for genotoxicity endpoints which included (i) structure–activity relationships (SAR) and (ii) quantitative structure–activity relationships ((Q)SAR) models for those components was performed by the Panel. The (Q)SAR analysis was performed using the OECD QSAR Toolbox. A summary of the results is reported in Appendix B.

The application of the OECD QSAR ToolBox did not identify alerts for DNA binding and carcinogenicity in any of the molecules (corresponding to the dimer I, II and III) analysed. An alert for in vivo genotoxicity (H-acceptor-path3-H-acceptor) was identified by one profiler (in vivo MN by ISS) for the three dimers. This alert is related to the capacity to form non-covalent binding with DNA and/or proteins as the result of the presence of two bonded atoms connecting two hydrogen bond acceptors. However, this alert is known to be of low positive predictivity (Benigni & Bossa, 2008) and therefore considered of low relevance. In addition, the protein binding alert for chromosomal aberration (CA) by the OASIS profiler, in particular Michael-type addition to activated double bonds in vinyl pyridines, was identified. Considering that (i) double bounds present in the vinyl pyridines of the three analysed molecules are embedded in the ring and hence not reactive and (ii) many carboxylic moieties are present in the molecules which can act as detoxifiers, the reaction underlying the alert is considered unlikely.

Overall, based on the available in silico data, the Panel considered that no concern for genotoxicity of the low molecular weight components (dimer I, II and III), which are present in the proposed food additive jagua (genipin-glycine) blue, is expected.

3.4.2.3 | *Genotoxicity of genipin*

Based on the proposed specifications provided by the applicant (see Section 3.1.2), genipin, the starting material used for the production of jagua (genipin-glycine) blue, can be present in the final product as a residual impurity at levels < 10 mg/kg.

The Panel considered some studies retrieved in the literature (Hobbs et al., 2018; Ozaki et al., 2002; Tsai et al., 2000) investigating the potential genotoxicity of genipin. In addition, upon request from EFSA, a newly generated study by the applicant was evaluated (Documentation provided to EFSA No. 5). For a detailed summary of the studies, see Appendix C.

Tsai et al. (2000) reported an in vitro micronucleus assay and a sister chromatid exchange (SCE) assay both in CHO-K1 cells. In the first assay, cells were treated with 0, 1, 10, 50 μg/mL of genipin for 6 h with and without metabolic activation (S9 mix). The positive controls used were cyclophosphamide (2.0 μg/mL) and mitomycin C (0.001 μg/mL) with or without metabolic activation. 1000 binucleated cells were scored for micronuclei. MTT assay was used to determine the cytotoxicity. No increase of the frequency of micronuclei was observed at any concentration. In the SCE assay, cells were treated with the same concentrations of genipin as in the in vitro micronucleus assay and the same test conditions were used. There were no significant differences in the number of SCE in the genipin-treated cells compared to the negative control.

The Panel noted that the MTT assay is not adequate to properly assess cytotoxicity in an in vitro MN assay. At the maximum concentration tested the mitotic index (the adequate parameter of cytotoxicity) was more than 80%, while the OECD TG 487 recommends to reach a 50%–60% cytotoxicity. The SCE assay is not validated for regulatory purposes.

Therefore, the Panel evaluated this study not reliable and of low relevance and did not consider it further in the assessment.

Ozaki et al. (2002) reported several studies with the aim to evaluate the genotoxicity potential of Gardenia yellow, a natural colourant extracted from the gardenia fruit, and its components, including genipin. Genipin was tested in A bacterial reverse mutation test, a *rec*-assay and a SCE assay. The bacterial reverse mutation assay was performed in *S. typhimurium*

TA98 and TA100 only and gave negative results. The Panel considered this study as reliable with restriction and of limited relevance since not performed according to GLP and deviating from the specific OECD TG 471 with the use of only two strains out of the ones recommended. The rec-assay and SCE assay reported positive results, however the Panel noted that these assays are not validated for regulatory purposes. Overall, the Panel evaluated these studies as not reliable and of low relevance and did not consider them further in the assessment.

Hobbs et al. (2018) evaluated the genotoxic potential of genipin through a GLP-compliant test battery conducted according to OECD TGs and the studies are described below. In particular, genipin was tested both in vitro and in vivo.

In a bacterial reverse mutation assay, genipin was tested in *S. typhimurium* TA97a, TA98, TA100, TA1535 and *Escherichia coli* WP2 uvrA pKM101 at concentrations of 0, 2, 6.4, 20, 64, 200, 640, 2000, 5000, 6000 μg/plate (±S9, pre-incubation method), in compliance with OECD TG 471 (OECD, 1997c). The study authors consider this test as equivocal, because of an apparent concentration-response trend observed only in TA97a. However, the Panel noted that the maximum fold-increase in revertants was less than 1.7, which is usually considered not biologically relevant. Therefore, the Panel considered this study as clearly negative, reliable without restrictions and of high relevance.

An in vitro micronucleus assay was performed using human TK6 cells which were treated with genipin at concentrations of 0, 2.5, 5, 10, 25, 50, 75 μg/mL for the short-term treatment with metabolic activation (4h, +S9) and at 0, 4, 6, 8, 10 μg/mL for the long-term treatment without metabolic activation (24h, −S9). The assay was conducted in compliance with OECD TG 487 (OECD, 2010) and subsequent updates (OECD, 2014). In the short-term treatment with S9 a significant increase in micronuclei was reported only at 5 μg/mL, however the Panel noted that the value was within the historical control data (HCD); in the long-term treatment (24h, only −S9) significant increases were reported at 6 and 8 μg/mL, hence the study was considered clearly positive. The Panel considered this study reliable without restrictions and of high relevance.

An in vitro mammalian chromosomal aberration test was performed using human CHO-WBL cells which were treated with genipin at concentrations of 0, 10, 25, 50, 75 μg/mL for the short-term treatment with and without metabolic activation (4h, ±S9) and at 0, 5, 10, 25, 50 μg/mL for the long-term treatment only without metabolic activation (20h, −S9). The assay was conducted in compliance with OECD TG 473 (OECD, 1997a) and subsequent updates (OECD, 2014). The Panel considered this assay of reliable without restrictions and of high relevance and clearly positive with and without metabolic activation at several concentrations. Also, polyploidy was increased at one or more concentrations under all treatment conditions. It is noted that polyploidy may reflect mitotic spindle disturbance, that is a possible cause of aneuploidy, although it can be also the consequence of cytotoxicity.

In addition, an in vitro comet assay and an in vitro reverse comet assay in human TK6 cells were performed, with equivocal results as reported by the study authors; however the Panel noted that, at the moment, no test guideline is available for these assays and considered both studies of low relevance.

The positive results observed in vitro in the micronucleus and chromosomal aberration assays were followed up in vivo with a combined micronucleus/comet assay.

This study was performed in B6C3F1 mice, both in males and females, and genipin was administered to the animals by gavage at doses of 8, 25, 74 and 3 (only in the vivo micronucleus assay), 8 (only in the vivo micronucleus assay), 25, 74, 222 mg/kg bw per day in males and females, respectively. The exposure to genipin were at doses reaching the maximum tolerated dose (74 and 222 mg/kg bw per day for males and females, respectively). In the in vivo micronucleus assay, a positive finding was observed in males at 74 mg/kg bw per day, the highest dose tested (at which two out of four animals had died). This observation was explained by the study authors as associated with the overt toxicity observed in this group and in particular driven by one single animal, which was considered to be an outlier. The authors noted that, with the exclusion of this animal, the mean MN-RET is comparable to those of the other groups. According to the study authors, the study should be considered as equivocal. The authors decided to repeat the experiment in females, that appeared more tolerant to the toxic effects of the test item. In this experiment, a slight statistically significant positive trend test, but dose group significantly differed from the negative control. According to the study authors, this outcome was largely driven by the animals of the 222 mg/kg bw per day dose group, the highest dose tested, exhibiting overt signs of toxicity. Overall, the study authors considered the assays as negative.

In the in vivo comet assay, liver, duodenum and stomach were analysed. Liver was analysed in both sexes while duodenum and stomach only in females. The Panel noted that this study was performed before the publication of the current OECD TG 489. Overall, negative results were observed in liver in males; in females, a statistically positive increase of DNA damage in liver at the lowest dose group (25 mg/kg bw per day) was observed, but not at any of the higher doses tested.

The Panel considered these studies reliable with restriction and the results of limited relevance.

Taking into account all the studies available, the Panel considered that genipin is not mutagenic in two bacterial reverse mutation assays with and without metabolic activation (Hobbs et al., 2018; Ozaki et al., 2002). In vitro, genipin was demonstrated to be able to induce chromosomal aberrations in an in vitro micronucleus and a chromosomal aberration assay (Hobbs et al., 2018). Regarding the available in vivo studies, positive results were observed both in males and females in a combined micronucleus/comet assay (Hobbs et al., 2018). Although these results might be explained as due to secondary effects of the overt toxicity observed in the treated animals and could be considered not biologically relevant due to absence of a dose–response, the Panel evaluated both assays as equivocal due to some limitations and deviations from the relevant OECD TGs (e.g. the total number of male animals analysed was below the minimum of 5 recommended in the OECD TG 474, if also considering an animal as outlier; the total number of cells analysed in the in vivo comet assay deviates from the currently recommended in the OECD TG 489).

Therefore, in order to rule out the potential concern for in vivo genotoxicity of genipin, the repetition of an in vivo combined bone marrow micronucleus test and comet assay was requested to the applicant to be performed in stomach/duodenum and in liver via oral route (via gavage) in males and females, in accordance with OECD TGs 474 and 489. In case of a positive result in the in vivo micronucleus test, centromere analysis was also requested.

Genipin was therefore assessed for its genotoxicity in an in vivo micronucleus test and comet assay in Swiss albino mice (Documentation provided to EFSA No. 6). The study was performed according to GLP and in line with the OECD TGs 474 and 489 (OECD , 2016a; OECD , 2016b). The animals were tested up to the maximum tolerated dose (MTD), established by a preliminary dose range finding experiment, that were 112 mg/kg bw per day for males and 56 mg/kg bw per day for females. Genipin was administered by gavage to 6 mice/sex/group, once a day for three consecutive days. Animals were checked for signs of toxicity during the treatment period. On day 3 of the study, all animals were euthanised. The Panel considered this study reliable without restrictions and the results obtained of high relevance.

In the bone marrow toxicity test, at least 500 erythrocytes per sample were analysed to determine the polychromatic erythrocyte (PCE)/total erythrocytes (TE) ratio. A dose-dependent decrease was seen in genipin-treated mice. At the MTD, this decrease was 29.3% in males and 28.9% in females, providing sufficient evidence of bone marrow exposure. For the micronucleus assay, at least 4000 PCEs per animal were evaluated. Genipin did not cause a statistically significant increase in micronucleated PCEs (MNPCEs) at any dose, while the positive control group showed a clear increase.

Liver, glandular stomach and duodenum were collected separately for the comet assay and histopathology. Genipin did not cause statistically significant DNA strand breaks in any of the tested tissues, indicating no genotoxic activity under the study conditions.

In conclusion, under the experimental conditions reported in the literature, in vivo genipin exposure did not result in a statistically significant increase in MN formation in PCE in bone marrow and did not induce statistically significant DNA strand breaks in cells from the glandular stomach, duodenum and liver cells at any of the tested doses . Therefore, the Panel considered that genipin does not raise a concern regarding genotoxicity.

3.4.2.4 | *Conclusion on the genotoxicity of jagua (genipin-glycine) blue*

Overall, taking into account the negative results on the experimental data on jagua (genipin-glycine) blue and genipin, the (Q)SAR analysis on the low molecular weight components, the Panel concluded that the proposed food additive does not raise a safety concern for genotoxicity.

3.4.3 | Acute toxicity

An acute oral toxicity study (Limit Test) (jagua extract, concentration of blue polymer 33.05%[9]; vehicle: sterile water) performed according to OECD TG 420 (OECD, 2002) was submitted by the applicant (Documentation provided to EFSA No. 1).

Five female Wistar (Crl: WI(Han)) rats were administered a single dose of 2000 mg/kg bw jagua extract by gavage. No mortality, clinical signs, effects on body weight or general toxicity were observed for 14 days after treatment.

3.4.4 | Short-term and sub-chronic toxicity

Rat

A dose range finding (DRF) study and a 90-day toxicity study in rats were submitted by the applicant (Documentation provided to EFSA No. 1). Both studies were performed in compliance with GLP.

The DRF study was performed as a 28-day oral toxicity study in male and female Wistar (Crl:WI(Han)) rats with jagua extract (concentration of blue polymer 33.05%[8]; vehicle: sterile water). The animals were randomly assigned to six dose groups (3 rats/sex/group) and administered by gavage 0, 10, 50, 100, 500 or 1000 mg/kg bw per day jagua extract. No mortality and no effects on body weight, body weight gain and food consumption were observed. Transient clinical signs (slight to severe piloerection, moving the bedding) were attributed by the authors to a local reaction to jagua extract, since these findings were observed immediately following the administration of the test substance. Slight changes in haematological and clinical biochemistry parameters were observed and not considered treatment related. At the end of the treatment period, no toxicological relevant effects on organ weights (brain, liver, heart, adrenal glands, spleen, testes, prostate and ovaries) were observed. The only macroscopic findings were a dark discoloration in kidneys in females and males of the highest dose group. However, no other kidney-related changes (size, consistency, serum markers) were observed. A dark discoloration was also observed in testes and epididymis in males of the highest dose . These findings were considered to be related to the intense colour of the test substance but not adverse. In conclusion, in this DRF study no adverse effects were observed up to the highest dose tested, i.e. 1000 mg/kg bw per day.

The 90-day toxicity study was performed in Wistar (Crl:WI(Han)) rats with jagua extract (concentration of blue polymer 33.05%[8]; vehicle: sterile water), according to OECD TG 408 (OECD, 1998). The animals (10 rats/sex/group) were treated by

[9]As reported in the related certificate of analysis (CoA) (Documentation provided to EFSA No. 3).

gavage with 0, 100, 300 or 1000 mg/kg bw per day of jagua extract for 90 days. The study included a 28-day recovery period (5 animals/sex of the control and high dose groups only). No mortality was observed. Clinical observations included discoloration of faeces observed in all treated animals, also during the recovery period (unexpectedly also in 3/5 control females). Alopecia and crust were observed as minor and transient clinical signs (mainly in high dose females). In the functional observation battery (FOB), no test substance-related effects were reported. At study day 90, slightly lower body weights compared to controls were observed in males of the low dose group (–10%) and the high dose group (–5%) as well as in males of the high dose group at the end of the recovery period (–8%); these changes were not statistically significant. The mean daily weight gain in males compared to controls was reduced non-statistically significantly in the low dose group (–18%) and in the high dose group (–9%) as well as statistically significantly in males of the high dose group at the end of the recovery period (–29%). No reduction of body weight or body weight gain were reported for females. The finding was not considered to be toxicologically relevant by the study authors since recorded body weights were in the range of the HCD in all groups and this change was observed only in one sex. The Panel considered that the reduction of body weight gain in male rats at the end of the recovery period was substantial but in the absence of a dose–response of this effect during the treatment period – was not considered treatment-related. No changes in food consumption were observed in males or females. No treatment-related effects were observed in haematology and blood coagulation. Minor statistically significant findings in clinical biochemistry parameters only in high dose groups, i.e. a lower alanine phosphatase level in males and an increased urea level in females, were not considered of toxicological relevance. Gross pathology findings were reported in kidneys at the end of the treatment period, i.e. dark discolouration in both males (1/10, 4/10 for mid and high dose group, respectively) and in females (4/10 for high dose group). Regarding organ weight changes, statistically significant effects were reported for a reduction of adrenal weights relative to brain weight in high dose females (–17% compared to controls) along with non-significant reductions in relative (to body weight) adrenal weights (–15% compared to controls) and absolute adrenal weight (–17% compared to controls). Non-statistically significant reductions in absolute as well as relative (to brain weight) thyroid/parathyroid gland weights (–33% and –34% compared to controls, respectively) were observed in high dose males and also to a minor degree in high dose females (–8% and –12% compared to controls, respectively) at the end of the recovery period. Reductions in other organ weights of males at the end of the recovery period were less pronounced, e.g. reductions in absolute and relative (to brain weight) thymus weights (–25% and –26% compared to controls, respectively) and in absolute and relative (to brain weight) spleen weights (–17% and –18% compared to controls, respectively) at the highest dose. Other changes in organ weights, e.g. decreases in absolute kidney weight and an increased relative brain weight in males were not dose dependent. The Panel noted that the reported changes in organ weights were not associated with corresponding pathological or histopathological findings. The Panel identified a NOAEL of 1000 mg/kg bw per day, the highest dose tested.

Dog

A DRF study and a 90-day toxicity study in dogs were also submitted (Documentation provided to EFSA No. 1). Both studies were performed in compliance with GLP.

The DRF study was performed in males and females Beagle dogs. The animals were randomly assigned to four dose groups (3 animals/sex/group) and given by gavage with 0, 250, 500 and 1000 mg/kg bw per day jagua extract (concentration of blue polymer 33.79%; vehicle: sterile water) for 28 days. No mortalities or treatment-related effects in clinical observations, body weight, food consumption, ophthalmology, electrocardiography, haematology, clinical chemistry and urinalysis were reported. In addition, no treatment-related macro- and histopathological findings were observed. Blue faeces and green urine were reported in all animals of all treated groups. Concentrations of the blue polymer were measured in plasma samples after single and repeated administration (see Section 3.4.1). No detectable concentrations were found, but as mentioned above (see Section 3.4.1) the method used had inadequate sensitivity. In conclusion, in this DRF study no adverse effects were observed up to the highest dose tested, i.e. 1000 mg/kg bw per day.

A 90-day toxicity study was performed in males and females Beagle dogs. The compliance with the related OECD TG was not specified. The animals were randomly assigned to four dose groups (3 animals/sex/group) with 0, 250, 500 or 1000 mg/kg bw per day jagua extract (concentration of blue polymer 33.79%; vehicle: sterile water) and treated by gavage for 90 days. The Panel noted that the number of animals used in this study deviates from OECD TG 409 on repeated dose 90-day oral toxicity study in non-rodents, which recommends using 4 animals/sex/group. No mortality and no effects on body weight and food consumption were observed. No toxicologically relevant changes in clinical signs were noted, however blue faeces and urine were observed in all animals of the treated groups. No changes were recorded at ophthalmoscopy and at electrocardiography examinations. No toxicologically relevant changes were recorded in haematology, clinical chemistry and urinalysis. There were no macro- and histopathological treatment-related changes observed at any dose tested. No toxicological relevant effects on organ weights (brain, liver, heart, kidney, adrenal glands, spleen, testes, thymus and ovaries) were observed. No detectable concentrations of the blue polymer were measured in plasma samples after both single and repeated administration, but as mentioned above, (see Section 3.4.1) the method used had inadequate sensitivity. In conclusion, a NOAEL of 1000 mg/kg per day was identified by the authors of this study. The Panel agreed with this conclusion.

3.4.5 | Chronic toxicity and carcinogenicity

No chronic toxicity and carcinogenicity studies according to Tier 2 of the current 'Guidance for submission for food additive evaluation' (EFSA ANS Panel, 2012) were submitted.

However, the applicant submitted a 12-month dietary toxicity study in rat including in utero exposure, according to an in-house method and to the internationally accepted guideline from Redbook 2000: IV.C.8 In-Utero Exposure Phase for Addition to Carcinogenicity Studies or Chronic Toxicity with Rodents (2007) (Documentation provided to EFSA No. 1). This study was performed according to GLP

A palatability study was also performed before the start of the main toxicity study. Six female Wistar rats (3 animals/group) were exposed to jagua extract at a concentration of 0 or 50,000 mg/kg diet (equivalent to 6000 mg/kg bw per day) for 14 days. Clinical signs, mortality, body weight and food consumption were measured. No treatment-related effects were observed. Therefore, 50,000 mg/kg diet was the highest concentration used for the main study.

In the main study, 20/sex/group Wistar rats (RccHanTM: WIST, age 6–7 weeks at the start of the study) were administered with jagua extract (concentration of blue polymer 36.4%) at a concentration of 0, 2500, 12,500 or 50,000 mg/kg diet (equal to 0, 181, 851 or 3439 mg/kg bw per day for F0 males during the premating phase and 0, 172, 857 or 3357 mg/kg bw per day for F0 females during the premating period; during the gestation period, the test substance intake was equal to 0, 220, 116 or 4155 mg/kg bw per day; and during the lactation period days 1–14: 0, 478, 2377 or 9024 mg/kg bw per day, respectively). Administration of the test substance in the diet was for 10 and 4 weeks prior to mating for males and females respectively, and for 3 weeks during mating. Males were subsequently treated for 6 weeks post-mating up to termination after weaning; females were treated during pregnancy, lactation and through weaning. From the offspring generation (F1), 2 pups/sex/litter/group were selected at weaning and 25 animals/sex/group were selected randomly for the next generation. F1 generation were subsequently orally (diet) exposed to the test item at the beginning of weaning and throughout the study for 52 weeks. The same concentrations administered to the parental generation (F0) were used, i.e. 0, 2500, 12,500 or 50,000 mg/kg diet (equal to 0, 168, 831 or 3385 mg/kg bw per day and 0, 187, 889, 3750 mg/kg bw per day in males and females, respectively).

Body weight, food consumption, clinical signs, FOB tests, organ weight, gross necropsy and histopathology were examined in both generation F0 and F1. All animals of F1 generation also underwent ophthalmological examination. In addition, changes in haematological and clinical biochemistry parameters, blood coagulation and urinalysis were measured for all animals of F1 generation. Only organ weights for kidney and liver were measured for the F0 generation. F0 females were further examined for litter health indices, precoital interval, gestation duration, reproductive indices, pup survival and pup external abnormalities.

No mortalities were observed in the F0 generation. Among the clinical observations, discoloured faeces were reported in all treated F0 males and females at terminal sacrifice. In addition, low incidences of clinical signs like crust, piloerection and alopecia without clear dose–responses were observed. In F0 males and females, some statistically significant findings were observed in the FOB which are considered of minor toxicological relevance. Regarding body weight changes in F0 animals, it was noted that the decreases were only minor and not toxicologically relevant. For body weight gains, there was a small but statistically significant decrease in high dose males during premating day 1–8 (–14%) and non-statistically significant decreases also later in life (e.g. –12% at day 57–64) and for high dose females a substantial but non-statistically significant reduction in body weight gain (–48%) was observed at the end of the premating period (day 22–29) however less pronounced in the gestation period (–8%). These effects may be partly explained by a lower food consumption in males and females. However, in the preceding 14-day palatability study with the test substance (0–50,000 mg/kg diet) in female rats, no effects on body weight and food consumption were detected. Some pathological findings without a clear dose–response were observed in males, i.e. enlarged livers, blue discolouration of kidneys in the high dose group only (with an incidence of 16/20), enlarged spleen (2/20, 3/20 and 1/20 in the low, mid and high dose groups, respectively) and red foci in thymus (controls and treated males). In treated females, findings were reported in liver (isolated effects) and kidney (blue discolouration in the high dose group with an incidence of 17/20; cyst in one mid dose female). Liver and kidney weights from F0 animals were not affected. The study authors suggested that the blue discoloration could be an '*observational artefact*', due to the researchers' knowledge of the blue colour displayed by the test item. However, the study authors could not exclude that some breakdown products of the proposed food additive could be systemically available and therefore be responsible for the discoloration. No histological correlation was observed in the kidneys. The Panel agreed with the authors that the blue discoloration observed in the kidney was not adverse because it was not accompanied by histopathological findings.

During the post-weaning phase of the F1 animals, four males were sacrificed prematurely in moribund conditions, two of them from high dose group (at 32 or 34 weeks), for which the cause of morbidity was considered to be malignant lymphoma (incidence: 2/25). In addition, four females (three from the mid dose group and one from the low dose group) were sacrificed prematurely for various reasons. Upon EFSA request for historical control data (HCD) on malignant lymphoma, the applicant provided a pathologist's statement reporting that malignant lymphoma is a common tumour in this strain of rats (Documentation provided No. 3). A mean incidence of 3.29% in males was reported in the publication by Weber (2017). The pathologist concluded that this type of tumour is not uncommon in young adult rats and an increase in incidence is usually seen with increasing age. Supportive data can be also found in Son et al. (2010) that reported malignant lymphoma as the most frequently occurring tumour in rats up to 52 weeks with an incidence of < 0.2% and of 0.5% at 35-weeks and in Envigo, 2016 with an incidence range of 0%–12% at 104-weeks.

The Panel agreed that this type of tumour is not uncommon in young adult rats. Also, the Panel noted that no pre-stages of lymphomas were observed. However, the Panel noted that control incidence data provided were coming mainly from 2-year toxicity studies (from 1981 to 2012, Weber, 2012 and Weber, 2017) which are not fully comparable with the exposure foreseen in the study under evaluation (12-months including in utero exposure) and that no proper characterisation of the tumours was done to substantiate the dismissal of this finding.

Upon an additional request from EFSA, the applicant provided further elaborations on the biological plausibility of the observed tumours and further reasons for dismissing the occurrence of these lymphomas. A peer-review of the study by a second pathologist was provided (Documentation provided to EFSA No. 5). The pathologist confirmed that the cause of morbidity of the two F1 males was a multicentric malignant lymphoma, likely originating from the spleen or bone marrow. The lymphomas were thoroughly described and considered to correspond to the pattern for spontaneously arising immunoblastic malignant lymphomas in Wistar Han rats.

Absence of morphological perturbation of lymphoid tissue morphology, suggesting a lack of immuno-suppressive or immuno-stimulatory potentials, were confirmed by the peer-review, which corroborated that the observed tumours were not treatment-related. The pathologist, therefore, concluded that the two lymphomas were spontaneous in nature.

Taking into account these further explanations and the absence of genotoxicity of jagua (genipin-glycine) blue, the Panel considered that the observed malignant lymphomas were not likely to be treatment-related.

Similarly to the parental generation, low incidences of clinical signs like crust, piloerection and alopecia without clear dose–responses were observed in the F1 generation. In addition, discoloured faeces were reported in all treated F1 males and females throughout the study period. In some F1 male and female animals (from PND 21 to the end of the study) of the high dose group only '*greenish*' urine was reported. No relevant effects were observed during ophthalmological examination in F1 animals. In males and females, some statistically significant findings were observed in the FOB which are considered of minor toxicological relevance by the Panel. Regarding the mean body weight change, statistically significant decreases and increases were observed in males and females of all treated groups. Considering the overall period (days 1–344), no statistically significant changes were observed in any of the treatment groups. For mean body weight gains, no statistically significant changes were observed in both sexes. Slight statistically significant decreases in food consumption were observed in both sexes in the mid and high dose groups. The Panel considered that these effects are not adverse due to lack of consistency and dose–response.

No adverse or toxicologically relevant treatment-related changes were observed for haematological and clinical biochemistry parameters. No treatment-related effects were observed in urinary parameters.

Regarding the organ weights, a slight but statistically significant decrease of absolute heart weight in females of the high dose group was observed, however not accompanied by histological findings. Therefore, the Panel considered these changes as not adverse. No other relevant effects on organ weights were reported. At necropsy, in F1 animals of the mid and high dose groups blue discoloration of gastrointestinal segments (stomach, duodenum, jejunum, ileum, cecum, colon) was observed (1/25 and 4/25, respectively). These effects were not accompanied by any histological changes. The study authors considered the reported blue discoloration of gastrointestinal segment as treatment-related but transient and due to faeces discoloration by the test item. The Panel agreed with this conclusion and considered these effects as not adverse.

The developmental and reproductive endpoints of this study are described in Section 3.4.6.

Overall, the Panel considered that the blue discoloration observed in kidneys of F0 animals and in the GI segments of F1 animals are not adverse, given that no histological findings were identified in either case. The Panel considered that, based on the further clarifications provided by the applicant and on the lack of treatment-related changes in any other organs, the malignant lymphomas observed in two males of the F1 generation were likely not treatment-related. Therefore, the Panel identified a NOAEL of 50,000 mg jagua (genipin-glycine) blue/kg diet (equal to 3439 and 3357 mg/kg bw per day in F0 males and females and 3385 and 3750 mg/kg bw per day in F1 males and females, respectively), the highest dose tested.

3.4.6 | Reproductive and developmental toxicity

No reproductive and developmental toxicity studies were submitted.

However, a 12-month dietary toxicity study including in utero exposure was provided by the applicant (see Section 3.4.5). No statistically significant effects were observed in litter parameters, including total number of pups born, number of male pups, number of female pups, sex ratio, number of live pups, stillbirths and runts during postnatal days 0–21. In addition, no treatment-related effects were observed on pup mean weight, total litter weight, male and female litter weight and mortality of pups between postnatal days 0 and 21. An increased non-statistically significant precoital interval time was observed in the mid dose group; however, no dose-dependence was observed. No effects were observed in the duration of gestation. No treatment-related adverse effects were observed in percentage copulation, fertility, delivery, number of corpora lutea, number of implantation sites and percentages of pre- and post-implantation loss. No effects were observed in the viability index of pups during postnatal days 0–4. In pups, some external findings (e.g. dark snout, oedema on the neck and absent tail tip) were observed in a few pups in the different groups, including control. Regarding pup gross external abnormalities, no toxicological relevant effects were observed. No effects on reproductive and developmental endpoints were observed up to the highest dose tested. The Panel identified a NOAEL of 50,000 mg jagua (genipin-glycine) blue/kg diet (equal to 3439 and 3357 mg/kg bw per day in F0 males and females, respectively).

3.4.7 | Allergenicity

In relation to allergenicity of the proposed food additive, the applicant submitted a repeated insult (occlusive) patch test with the aim to determine the irritation and/or sensitisation potential of jagua (genipin-glycine) blue after repeated application to the skin of 52 human volunteers with self-perceived sensitive skin (Documentation provided to EFSA No. 1). No skin irritation or allergic contact dermatitis was reported. The Panel did not consider this study for the safety assessment of the proposed food additive, since its relevance to oral allergenicity of jagua (genipin-glycine) blue remains unclear.

Following an additional data request, the applicant performed a literature search on genipin and *G. americana* (source plant of jagua fruit) in order to identify evidence related to potential allergenicity (Documentation provided to EFSA No. 2). As a result, no references on allergenicity by oral intake of genipin or *G. americana* (source plant of jagua fruit) were retrieved. However, four publications reporting on contact dermatitis following dermal exposure (topical, tattoo purpose) to solutions/gels containing *G. americana* extracts were found (Bircher et al., 2017; Stout et al., 2019; Waton et al., 2017; Wilmot & Wakelin, 2020). In these papers (except Waton et al., 2017), patch testing was performed and indicated genipin as causative allergen.

The Panel noted that in the case-reports retrieved by the applicant contact dermatitis was related to dermal genipin exposure.

Concerning the new proposed food additive itself, the Panel found no case-reports on food allergy and/or food intolerance to jagua (genipin-glycine) blue in the literature.

The applicant also reported that internationally recognised databases of known and/or putative allergens (i.e. WHO/IUIS, AllergenOnline, Allergome) were used to retrieve information on proteins derived from *G. americana* in relation to their potential allergenicity and that no records were found. In addition, the applicant provided a bioinformatic analysis using UniProt KB database. In the database, proteins identified in the source plant *G. americana* of the proposed food additive (and further sequenced) were searched. The results showed that 22 proteins, listed with FASTA sequences, were found. In NCBI Protein database, 30 proteins from *G. americana* origin were identified and their FASTA sequences were provided by the applicant. A comparison of the FASTA sequences to the entries of the AllergenOnline database (2233 peer reviewed amino acid sequences) was performed. The results showed that none of the previous mentioned proteins showed matches with a > 35% identity over 80-amino acid length sequences in the AllergenOnline FASTA searches or showed > 50% alignment with known allergens in this database (Documentation provided to EFSA No. 3).

The Panel noted that the bioinformatics analysis performed were appropriate and no sequence identity greater than 35% to a known allergen using a sliding window of 80-amino acids was reported (EFSA CEP Panel, 2021; FAO/WHO, 2001).

Overall, the Panel considered that no oral allergenicity of jagua (genipin-glycine) blue is expected.

4 | DISCUSSION

The European Commission requested EFSA to provide a scientific opinion on the safety of the proposed use of jagua (genipin-glycine) blue as a food additive, in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

The proposed food additive, named as jagua (genipin-glycine) blue, is manufactured by water extraction of the ground pulp of the peeled, unripe fruits of *Genipa americana*, followed by filtration of the extract. The functional component, i.e. the blue polymer is formed through a reaction between genipin, an iridoid compound naturally present in the extract, and stoichiometrically added glycine (authorised food additive E 640). Specifically, this reaction leads to the formation of the blue-coloured polymer (referred to by the applicant as Compound 4) and minor colouring components (dimers, referred to by the applicant as Compounds 1, 2, 3). Jagua (genipin-glycine) blue consists of the blue polymer (20%–40%), the minor colouring compounds (< 0.4%), carbohydrates and proteins and is commercialised as a deep blue powder, containing a carrier (e.g. maltodextrin or modified starch). The Panel emphasises that the blue colour is attributed mainly to the blue polymer and less to minor blue colouring components. In the proposed specifications of jagua (genipin-glycine) blue, a limit of 10 ppm is included for residual genipin, a well-known protein cross-linker (Wahba, 2024). The analytical data submitted indicated that genipin was below the LOQ of 10 ppm in all analysed samples.

Regarding the proposed specifications, the Panel would suggest a more detailed description of the manufacturing process of the proposed food additive in the Definition. Specifically, it should be noted that the content of genipin in the unripe fruits is calculated, and stoichiometric amounts of glycine are added, for the blue polymer to be formed. A carrier is also added to prepare the final powder. The Panel noted that the CAS Number proposed in the specifications by the applicant (1314879-21-4) refers to the blue polymer. The chemical names proposed refer to the reactants (genipin and glycine) and not to the blue polymer. In addition, the Panel noted that the proposal to include 'This reaction occurs naturally in the unripe fruits after being cut producing a mixture of blue compounds' in the Definition of the proposed specifications is not relevant to the manufacturing of the proposed food additive and is not necessary.

The Panel noted that the information provided on different batches of the proposed food additive showed that jagua (genipin-glycine) blue is produced according to the proposed specifications.

In addition, the Panel considered that the proposed specification range of 140%–280% for the colour *E10%* parameter is wide, compared to the analytical data provided (243%–273%), while the proposed specification limits for total fat of < 10% and for yeasts and moulds of below 100 CFU/g are higher than the analytical data provided (0.31%–1.99% for total

fat and < 10 CFU/g for yeasts and moulds). The Panel also noted that the total carbohydrate contents in the proposed food additive were calculated by the applicant by difference (100% – protein % – fat % – moisture % – ash %), without considering the functional component (blue polymer = 20% to 40%, according to the specifications), the minor blue colouring components and other components.

Despite the botanical origin of the proposed food additive, the Panel considered that no limits for pesticides in the proposed specifications of jagua (genipin-glycine) blue are needed, as long as it is assured that the fruits are collected from plants grown in low-risk areas for incidental contamination by pesticides or plants not commercially cultivated. Should the proposed food additive be prepared in full or in part from cultivated *G. americana*, limits for pesticides should be considered for inclusion in the EU specifications.

Analytical data on the levels of As, Pb, Cd and Hg were provided by the applicant for five samples of the proposed food additive. The Panel assessed the risk that would result if these toxic elements were present in jagua (genipin-glycine) blue according to two concentration scenarios: (i) considering their presence at the proposed specification limits and (ii) considering the data reported at the rounded up highest measured value (i.e. for Pb) and, in the absence of any measured value, at the reporting limit (i.e. for As, Cd, Hg), modulated by the Panel by applying a factor of 10 to allow flexibility with respect to representativeness, homogeneity and differing analytical methods. In the case of iAs, the Panel noted that its presence at the proposed specification limit value would lead to an MOE of 2.2. The Panel recommended to lower the specification limits proposed by the applicant for all four toxic elements (Pb, Cd, iHg, iAs), taking into account (i) the results of the calculations performed by the Panel (the MOE for iAs is close to 1 at which risk cannot be excluded), (ii) the fact that the proposed food additive is not the only potential dietary source of toxic elements and that (iii) the maximum limits should be established based on actual levels in the commercial food additive.

Regarding the particle size, the applicant provided results from SEM–EDX analysis on five batches of jagua (genipin-glycine) blue showing that for all batches the % of particles with one dimension smaller than 250 nm ranged from 73% to 98%, the median size of the particles ranged from 76 to 116 nm and 10% of particles ranged from 40 to 48 nm. The Panel concluded that, based on the data provided, the presence of small particles including nanoparticles in jagua (genipin-glycine) blue as produced is confirmed. However, the Panel could not assess if the identified particles belong to the blue polymer only and/or to the other components (sugars, carbohydrates, proteins, small amounts of ashes and modified starches) of the proposed food additive. Taking into account that these components are natural constituents of the starting material, which is the jagua fruit apart from the carrier and that the Panel identified that the blue polymer is the component of potential toxicological concern, the Panel considered that the particulate matter originating from the fruit itself or the carrier is not of interest for this risk assessment.

The applicant provided results of a water solubility test for jagua (genipin-glycine) blue powder conducted at 20°C using the OECD TG 105 shake flask method and 10 kDa ultrafiltration membrane, applying substance specific methodology to determine the concentration of the blue polymer. The water solubility of the blue polymer was approximately 6 g/L, though the applicant noted that material loss during filtration (up to two-thirds) likely led to an underestimation. Based on the data provided, the Panel considered that the water solubility of the blue polymer is at least 6 g/L and that it should be described as 'slightly soluble' in the proposed specifications.

The Panel noted that the maximum use levels of jagua (genipin-glycine) blue proposed by the applicant do not exceed 5000 mg/kg. Considering the content of blue polymer present in the proposed food additive to be 20%–40%, the concentration of the blue polymer in food would not exceed 2000 mg/kg. Taking into account the reported solubility of the blue polymer, the maximum proposed use levels 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 the blue polymer 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 of the blue polymer, in the proposed food additive at the proposed uses and use levels. Jagua (genipin-glycine) blue used as a food additive can therefore be assessed following the conventional risk assessment, i.e. EFSA Guidance for submission for food additive evaluations (EFSA ANS Panel, 2012).

A series of stability studies under normal and accelerated conditions were conducted to assess the stability of jagua (genipin-glycine) blue both in its powder form and when added to foods of the proposed uses (e.g. dairy and confectionery products, snacks and cereals, dairy substitutes, savoury snacks, candy, jelly). The Panel considered jagua (genipin-glycine) blue to be stable as a powder and in food matrices under the evaluated conditions.

The Panel acknowledged that the in vitro data provided by the applicant, i.e. an in vitro Caco-2 cell permeability test, demonstrated a low permeability of jagua (genipin-glycine) blue. However, the Panel considered that evidence from the in vivo studies suggested at least some absorption, in particular, green-coloured urine observed in the dog studies (all animals of all treated groups) and in the 12-months toxicity study in rats (although sporadically and only in the high dose groups) together with discoloration of some organs (kidney, testes, epididymis) in the 28- and 90-day toxicity studies in rats. No detectable concentration of the blue polymer was measured in dog plasma samples of the 28- and 90-day toxicity studies; however, the Panel noted that the method used in these studies had inadequate sensitivity. The high molecular weight of jagua (genipin-glycine) blue (more than 6000 Da) suggested a low absorption of the polymer. Therefore, the Panel considered that it is likely the discoloration reported in the in vivo studies are due to the low molecular weight components that are part of the proposed food additive or due to possible breakdown products. Based on this data and on evidence of systemic absorption in the toxicity studies available, the Panel considered that some absorption of the proposed food additive has been demonstrated.

The toxicological data set comprised in vitro and in vivo genotoxicity studies, studies on acute toxicity, sub-chronic toxicity (90-day toxicity study in rat; 90-day toxicity study in dog) and a long-term toxicity study including in utero exposure (12-month dietary toxicity study in rat). Although no standard reproductive and developmental toxicity studies were provided, the 12-month dietary toxicity study with in utero exposure was considered sufficient by the Panel to cover these endpoints and to conclude on the safety of jagua (genipin-glycine) blue.

Regarding genotoxicity, taking into account the negative results on the experimental data on jagua (genipin-glycine) blue and genipin along with the (Q)SAR analysis on the low molecular weight components, the Panel concluded that the proposed food additive does not raise a safety concern for genotoxicity.

In the 12-month dietary toxicity study in rat including in utero exposure, malignant lymphomas were observed in 2/25 males of the F1 generation. Based on additional data provided by the applicant (see Section 3.4.5), together with the nature of jagua (genipin-glycine) blue and its lack of genotoxicity, the Panel considered that the tumours were not likely treatment-related. Therefore, the Panel identified a NOAEL of 50,000 mg jagua (genipin-glycine) blue/kg diet (equal to 3439 and 3357 mg/kg bw per day in F0 males and females and 3385 and 3750 mg/kg bw per day in F1 males and females, respectively), the highest dose tested. In the in utero phase of this study, no reproductive or developmental effects were observed.

Considering the available data set, the Panel derived an ADI of 34 mg jagua (genipin-glycine) blue/kg bw per day or 12 mg/kg bw per day expressed as blue polymer, based on a NOAEL of 3385 mg jagua (genipin-glycine) blue/kg bw per day (or 1232 mg blue polymer/kg bw per day, considering a blue polymer content of 36.4%) from the 12-month dietary toxicity study in rat including in utero exposure (F1 generation), applying the default uncertainty factor (UF) of 100.

Dietary exposure to jagua (genipin-glycine) blue and its blue polymer was estimated with DietEx using the proposed maximum and typical use levels of jagua (genipin-glycine) blue in 16 food categories. The use of DietEx allows for the selection of more detailed food categories and thus results in more refined exposure estimates.

At the proposed maximum use levels, the mean exposure to jagua (genipin-glycine) blue from its estimated use as a food additive approximately ranged from 0.2 mg/kg bw per day in the elderly to 9 mg/kg bw per day in toddlers. The 95th percentile of exposure ranged from 1 mg/kg bw per day in the elderly to 27 mg/kg bw per day in toddlers. The Panel noted that the mean and P95 estimates of exposure did not exceed the proposed ADI for jagua (genipin-glycine) blue in all population groups.

The same was true for the exposure to the blue polymer assuming a 40% content in the proposed food additive. Highest estimate, based on the proposed maximum use levels, was approximately 11 mg/kg bw per day, which was below the proposed ADI of 12 mg/kg bw per day expressed as blue polymer.

The main food categories contributing to the total mean exposure estimates for all population groups were FC 01.4 'Flavoured fermented milk products, including heat-treated products' and FC 06.3 'Breakfast cereals'. For children, adolescents and the elderly FC 03 'Edible ices' was also an important contributor to the exposure.

Overall, the Panel considered that the uncertainties identified resulted in an overestimation of the dietary exposure to jagua (genipin-glycine) blue and its blue polymer in European countries considered in the EFSA Comprehensive database at both the proposed maximum and typical use levels.

5 | CONCLUSIONS

Based on the available toxicity data set, the Panel established an ADI of 34 mg jagua (genipin-glycine) blue/kg bw per day or 12 mg/kg bw per day expressed as blue polymer.

Taking into account the exposure estimates, the Panel concluded there is no safety concern for jagua (genipin-glycine) blue as a food additive at the proposed uses and use levels.

6 | DOCUMENTATION AS PROVIDED TO EFSA

1. Application for the authorisation of the use of a product called 'Jagua (Genipin-Glycine) Blue' as a food additive in several food categories of Annex II to Regulation (EC) No 1333/2008. Technical dossier. November 2021. Submitted by Ecoflora Cares.
2. Additional information submitted by the Ecoflora Cares following a request from EFSA. August 2022.
3. Additional information submitted by the Ecoflora Cares following a request from EFSA. December 2022.
4. Additional information submitted by the Ecoflora Cares following a request from EFSA. June 2023.
5. Additional information submitted by the Ecoflora Cares following a request from EFSA. April 2024.
6. Additional information submitted by the Ecoflora Cares following a request from EFSA. August 2025.
7. Additional information submitted by the Ecoflora Cares following a request from EFSA. October 2025 (via email).
8. Additional information submitted by the Ecoflora Cares following a request from EFSA. October 2025 (via email).

ABBREVIATIONS

ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion

ANS Panel	Panel on Food Additives and Nutrient Sources added to Food
As	arsenic
B[a]P	Benzo[a]pyrene
BET	Brunauer–Emmett–Teller
BMDL	benchmark dose (lower confidence limit)
bw	body weight
CA	chromosomal aberration
CAS	Chemical Abstract Service
Cd	cadmium
CEP Panel	Panel on Food Contact Materials, Enzymes and Processing Aids
CFU	colony forming units
CoA	certificate of analysis
CONTAM Panel	Panel on Contaminants in the Food Chain
CPA	cyclophosphamide monohydrate
DAD	diode array detection
DRF	dose range finding
EMS	Ethyl methane sulfonate
FAIM	Food Additive Intake Model
FAO/WHO	Food and Agriculture Organisation/World Health Organisation
FC	Food Category
FCS	Food Categorisation System
FOB	functional observational battery
GC–MS	gas chromatography–mass spectrometry
GEF	global evaluation factor
GLP	Good Laboratory Practice
HBGV	health-based guidance value
HCD	Historical Control Data
Hg	mercury
HPLC	high-performance liquid chromatography
HPLC–DAD	high-performance liquid chromatography–diode array detection
HPLC–MS	high-performance liquid chromatography/mass spectrometry
HS	horse serum
ICP–MS	inductively coupled plasma mass spectrometry
IR	infra-red spectroscopy
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC–MS	liquid chromatography–mass spectrometry
LD	laser diffraction
LLOQ	lower limit of quantification
LOD	limit of detection
LOQ	limit of quantification
MMS	methyl methane sulfonate
MN	micronucleus
MOE	margin of exposure
MS	mass spectrometry
MS	modified starch
MTD	maximum tolerated dose
MYC	mitomycin C
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level
OECD	Organization for Economic Co-operation and Development
P_{app}	apparent permeability rate coefficient
Pb	lead
PCE	polychromatic erythrocyte
pH	potential of hydrogen
PSD	particle size distribution
QSAR	quantitative structure–activity relationship model
RP	reference point
RTE	ready-to-eat
RTG	relative total growth
SC	Scientific Commission
SCE	sister chromatid exchange
SEM	scanning electron microscopy

TC	total carbohydrates
TE	total erythrocytes
TG	Test Guideline
TOC	total organic carbon analysis
TWI	tolerable weekly intake
UF	uncertainty factor
ULOQ	upper limit of quantification
USP–NF	United States Pharmacopeia–National Formulary
VIN	vinblastine sulfate
VSSA	volume specific surface area

ACKNOWLEDGEMENTS

The Panel wishes to thank the following for the support provided to this scientific output: Aldo Benigni.

REQUESTOR

European Commission

QUESTION NUMBER

EFSA-Q-2021-00231

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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., Barat Baviera, J. M., Degen, G., … Ruggeri, L. (2025). Safety evaluation of jagua (genipin-glycine) blue as a food additive. *EFSA Journal*, *23*(12), e9738. https://doi.org/10.2903/j.efsa.2025.9738

APPENDIX A

Genotoxicity studies on jagua (genipin-glycine) blue

Test system	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/ comments	Relevance of test system/relevance of the study result	Reference
In vitro tests						
Bacterial Reverse mutation test *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA102 OECD TG 471 GLP	Experiment I: 31.6, 100, 316, 1000, 2500 and 5000 µg/plate (±S9, plate incorporation method) Experiment II: 31.6, 100, 316, 1000, 2500 and 5000 µg/plate (±S9, pre-incubation method) Vehicle and negative control: Distilled water Positive controls: TA100, TA1535: Sodium azide (10 µg/plate) TA98: 4-nitro-o-phenylene-diamine (10 µg/plate) TA1537: 4-nitro-o-phenylene-diamine (40 µg/plate) TA102: MMS (1 µL/plate) TA98, TA100, TA1535, TA1537 (+S9): 2-aminoanthracene (2.5 µg/plate) TA102 (+S9): 2-aminoanthracene (10 µg/plate)	Jagua Extract Batch No: 5313002 Concentration of the blue polymer: 33.62%, as reported in the related CoA	Negative Some toxicity was reported in experiment I only in TA 1537 at concentrations of 2500 µg/plate and higher without S9 and in the experiment II in different strains at 100 µg/plate and higher without S9 and at 2500 µg/plate and higher with S9	1 (Reliable without restriction)	High/high	Documentation provided to EFSA No. 1
Mammalian cell gene Mutation assay Mouse lymphoma L5178Y cells OECD TG 476 GLP	Experiment I: 250, 500, 1000, 2000, 3000, 4000, 4500 and 5000 µg/mL (4 h, +S9) 500, 1000, 2000, 2500, 3000, 3500, 4000 and 4250 µg/mL (4 h, −S9) Experiment II: 600, 1200, 3200, 3600, 3900, 4200, 4500 and 5000 µg/mL (24 h, +S9) 100, 250, 500, 1000, 2000, 2500, 3000 and 3250 µg/mL (24 h, −S9) Vehicle and negative control: RPMI + HS Positive controls: B[a]P (1.5 µg/mL, +S9) EMS (200 µg/mL and 300 µg/mL, −S9) MMS (8 and 10 µg/mL, −S9)		Negative	1 (Reliable without restriction)	High/high	

(Continues)

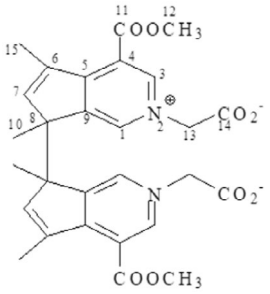
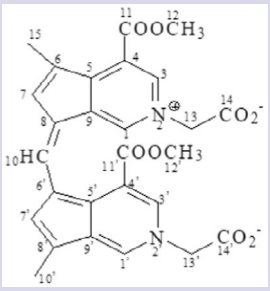
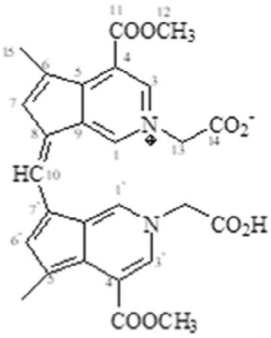
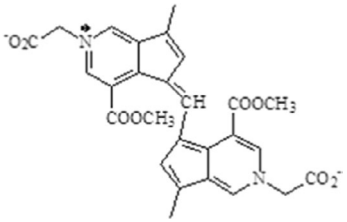
(Continued)

Test system	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/ comments	Relevance of test system/relevance of the study result	Reference
Micronucleus test Chinese Hamster Ovary (CHO-K1) cells OECD TG 487 GLP	500, 1000, 2000 µg/mL (3 h, ±S9) (3 h + 20 h, −S9) Vehicle and negative control: Water Positive controls: Cyclophosphamide (6 µg/mL, +S9) Mitomycin C (0.8 µg/mL, −S9) Vinblastine sulfate (0.08 µg/mL, −S9)	Jagua Extract Batch No.: 5322002 Concentration of the blue polymer: 33.47% polymer	Negative	1 (Reliable without restriction)	High/high	Documentation provided to EFSA No. 2
In vivo test						
In vivo micronucleus test Peripheral Blood 5 sex/group NMRI mice OECD TG 474 GLP	Intraperitoneal 400, 1000 and 2000 mg/kg bw 10 mL/kg bw 44 h: 400, 1000 mg/kg bw 68 h: 2000 mg/kg bw Vehicle: 0.9% NaCl Positive control: Cyclophosphamide (40 mg/kg bw)	Jagua Extract Batch No.: 5313002 Concentration of the blue polymer: 33.62%, as reported in the related CoA	Negative	3 (Not reliable) Inadequate route of administration	High/low	Documentation provided to EFSA No. 1

Abbreviations: B[a]P, benzo[a]pyrene; CAS, Chemical Abstract Service; CoA, certificate of analysis; DMSO, dimethyl sulfoxide; EMS, ethyl methanesulfonate; MMS, methyl methanesulfonate.

APPENDIX B

(Q)SAR analysis of minor colouring compounds of jagua (genipin-glycine) blue

Chemical name	CAS No.	Structure	QSAR ToolBox
Compound 1 7,7′-Bi-7*H*-cyclopenta[*c*] pyridinium, 2,2′-bis(carboxymethyl)-4,4′-(methoxycarbonyl)-5,5′,7,7′-tetramethyl-, bis, inner salt	1313734-13-2		DNA binding by OASIS: No alert found DNA binding by OECD: No alert found **Protein binding by OASIS:** • **Michael addition on polarised Alkenes** Protein binding by OECD: No alert found Carcinogenicity (genotox & non genotox): No alert found DNA alerts for Ames, CA and MNT by OASIS: No alert found In vitro mutagenicity (Ames) by ISS: No alert found **In vivo mutagenicity (Micronucleus) by ISS:** • **H-acceptor-path3-H-acceptor** Protein binding alerts for CA by OASIS: • **Michael-type addition to activated double bonds in vinyl pyridines**
Compound 2 5H-Cyclopenta[*c*] pyridinium,2-(carboxymethyl)-5-[[2-(carboxymethyl)-4-(methoxycarbonyl)-5-methyl-2H-cyclopenta[*c*] pyridin-7-yl]methylene]-4-(methoxycarbonyl)-7-methyl-,inner salt	104359-67-3		DNA binding by OASIS: No alert found DNA binding by OECD: No alert found **Protein binding by OASIS:** • **Michael addition on polarised Alkenes** Protein binding by OECD: No alert found Carcinogenicity (genotox & non genotox): No alert found DNA alerts for Ames, CA and MNT by OASIS: No alert found In vitro mutagenicity (Ames) by ISS: No alert found **In vivo mutagenicity (Micronucleus) by ISS:** • **H-acceptor-path3-H-acceptor** Protein binding alerts for CA by OASIS: • **Michael-type addition to activated double bonds in vinyl pyridines**
Compound 3 7*H*-Cyclopenta[*c*]pyridinium, 2-(carboxymethyl)-7-[[2-(carboxymethyl)-4-(methoxycarbonyl)-5-methyl-2H-cyclopenta[*c*] pyridine-7-yl] methylene]-4-(methoxycarbonyl)-5-methyl-, inner salt	1313734-14-3	Structure 3A 	DNA binding by OASIS: No alert found DNA binding by OECD: No alert found **Protein binding by OASIS:** • **Michael addition on polarised Alkenes** Protein binding by OECD: No alert found Carcinogenicity (genotox & non genotox): No alert found DNA alerts for Ames, CA and MNT by OASIS: No alert found In vitro mutagenicity (Ames) by ISS: No alert found **In vivo mutagenicity (Micronucleus) by ISS:** • **H-acceptor-path3-H-acceptor** Protein binding alerts for CA by OASIS: • **Michael-type addition to activated double bonds in vinyl pyridines**
		Structure 3B 	DNA binding by OASIS: No alert found DNA binding by OECD: No alert found **Protein binding by OASIS:** • **Michael addition on polarised Alkenes** Protein binding by OECD: No alert found Carcinogenicity (genotox & non genotox): No alert found DNA alerts for Ames, CA and MNT by OASIS: No alert found In vitro mutagenicity (Ames) by ISS: No alert found **In vivo mutagenicity (Micronucleus) by ISS:** • **H-acceptor-path3-H-acceptor** Protein binding alerts for CA by OASIS: No alert found

Abbreviations: CA, chromosomal aberration; CAS, Chemical Abstract Service; MNT, micronucleus test.

APPENDIX C

Genotoxicity studies on genipin

Test system	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/ comments	Relevance of test system/ relevance of the study result	Reference
In vitro tests						
Bacterial Reverse mutation test *Salmonella typhimurium* TA97a, TA98, TA100, TA1535 and *E. coli* WP2 uvrA pKM101 OECD TG 471 GLP	2, 6.4, 20, 64, 200, 640, 2000, 5000, 6000 µg/plate (±S9, pre-incubation method) Vehicle and negative control: DMSO Positive controls: TA100 (+S9): B[a]P (2 µg/plate) TA98 (+S9): 2-aminoanthracene (2 µg/plate) TA1535, TA97a (+S9): 2-aminoanthracene (2.5 µg/plate) WP2 uvrA (+S9): 2-aminoanthracene (20 µg/plate) TA100, TA1535: Sodium azide (1 µg/plate) TA98: 2-nitrofluorene (3 µg/plate) TA97a: ICR191 (0.25 µg/plate) WP2 uvrA: 4-nitroquinoline-N-oxide (0.25 µg/plate)	Genipin CAS No. 6902-77-8 Purity: 99.982%	Negative The authors consider this test as equivocal, because of an apparent concentration-response trend observed only in TA97a, but the increase maximum fold-increase was less than 1.7, usually considered not biologically relevant	1 (Reliable without restriction)	High/high	Hobbs et al. (2018)
Micronucleus test Human TP53 competent human lymphoblast TK6 cells OECD TG 487 GLP	250, 500, 1250, 2500, 5000 µg/mL (4 h + 20 h, +S9) 4, 6, 8, 10 µg/mL (24 h, −S9) Vehicle and negative control: DMSO Positive controls: Cyclophosphamide (12.5 µg/mL, +S9) Vinblastine sulfate (0.15 µg/mL, −S9)		Positive	1 (Reliable without restriction)	High/high	
Chromosomal aberration Chinese hamster ovary CHO-WBL cells OECD TG 473 GLP	10, 25, 50, 75 µg/mL (4 h + 20 h, +S9) 10, 25, 50, 75 µg/mL (4 h + 20 h, −S9) 5, 10, 25, 50 µg/mL (20 h + 4 h, −S9) Vehicle and negative control: DMSO Positive controls: Cyclophosphamide (5 µg/mL, +S9) Mitomycin C (0.15 µg/mL, −S9)		Positive	1 (Reliable without restriction)	High/high	
In vitro comet and in vitro reverse comet assay (for the detection of cross-linking agents) Human TP53 competent human lymphoblast TK6 cells GLP	2.7, 8.2, 25, 74, 222, 667, 2000 µM (4 h) 6.25, 12.5, 25, 50, 100, 200, 400 µM (24 h) Vehicle and negative control: DMSO DNA damage: styrene oxide (800 µM, 1 h). Positive control: EMS		Equivocal	3 (Not reliable) Assays currently not validated for regulatory purposes.	Low/low	

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Test system	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/ comments	Relevance of test system/ relevance of the study result	Reference
Bacterial Reverse mutation test *Salmonella typhimurium* TA98 and TA100	1, 0.5 and 0.25 mg/plate (±S9, pre-incubation method) Vehicle and negative control: DMSO Positive controls: TA98, TA100: 2-(2-furyl)-3-nitro-2-furyl) acrylamide (0.1 μg/plate) TA98, TA100 (+S9): 2-aminoanthracene (1 μg/plate)	Genipin Purity: NR	Negative Genipin showed toxicity at 0.5 mg and 1.0 mg/plate in strains TA98 and TA100 without S9mix, and at 1.0 mg/plate in strains TA98 and TA100 with S9 mix.	2 (Reliable with restriction) Overall compatible with OECD TG 471 (1997), but only two strains were tested	Limited/limited	Ozaki et al. (2002)
Rec-assay Recombinationless strain, M45 Rec- and the wild-strain, H17 Rec + of *Bacillus subtilis*	2.25 and 4.5 mg (24 h) Vehicle and negative control: DMSO Positive control: 2-(2-furyl)-3-nitro-2-furyl) acrylamide (0.015 μg)	Genipin Purity: NR	Positive	3 (Not reliable) Test currently not validated for regulatory purposes	Low/low	
Sister chromatid exchange (SCE) assay V79 Chinese hamster cells	2, 4, 6, 8 and 10 μg/mL Vehicle and negative control: DMSO	Genipin Purity: NR	Positive Linear relationship ($r = 0.98$, $p < 0.001$)	3 (Not reliable) Test not validated for regulatory purposes	Low/low	
Micronucleus test Chinese hamster ovary CHO-K1 cells	1, 10 and 50 μg/mL (6 h, ±S9) Positive controls: Cyclophosphamide (2 μg/mL, +S9) Mitomycin C (0.001 μg/mL, −S9)	Genipin Purity: > 98%	Negative	3 (Not reliable) Low concentrations tested (up to > 80% cytotoxicity)	Low/low	Tsai et al. (2000)
Sister chromatid exchange (SCE) assay Chinese hamster ovary CHO-K1 cells	1, 10, 50 μg/mL (6 h, ±S9) Positive controls: Cyclophosphamide (2 μg/mL, +S9) Mitomycin C (0.001 μg/mL, −S9)	Genipin CAS No. 6902-77-8 Purity: 99.982%	Negative	3 (Not reliable) Test not validated for regulatory purposes	Low/low	

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Test system	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/ comments	Relevance of test system/ relevance of the study result	Reference
In vivo tests						
In vivo micronucleus test Peripheral blood 6 ♂/group, 5 ♀/group B6C3F1 mice OECD TG 474 GLP	Oral Gavage 8, 25, 74, 222, 667 mg/kg bw/day (♂) 3, 8, 25, 74, 222 mg/kg bw/day (♀) 3 days Vehicle: corn oil Positive control: EMS (150 mg/kg bw/day)	Genipin CAS No. 6902-77-8 Purity: 99.982%	Equivocal In males, a positive outcome at the highest dose is apparently due to an outlier. In females, the trend is statistically significant, and two dosages were slightly over the HCD.	2 (Reliable with restriction)	High/limited	Hobbs et al. (2018)
In vivo comet assay Liver, 6 ♂/group Liver, duodenum and stomach, 5 ♀/group B6C3F1 mice OECD TG 489 GLP	Oral Gavage 8, 25, 74 mg/kg bw/day (♂) 25, 74, 222 mg/kg bw/day (♀) 3 days Vehicle: corn oil Positive control: EMS (150 mg/kg bw/day)		Equivocal	2 (Reliable with restriction)	High/limited	
In vivo reverse comet assay in liver Liver B6C3F1 mice, ♀	Oral Gavage 222 mg/kg bw/day genipin + styrene oxide (2.4 mM and 3.6 mM, 1 h)		Equivocal The authors considered the effect as not statistically significant, with $p = 0.0526$.	3 (Not reliable) Test not validated for regulatory purposes	Low/low	
Combined micronucleus and comet assay Bone marrow (MN) Liver, glandular stomach and duodenum (Comet assay) Swiss albino mice OECD 474 OECD 489 GLP	Oral Gavage 1st DRF: 31.25, 62.50, 125, 250 and 500 mg/kg bw/day (3 ♂, 3 ♀) 2nd DRF: 81, 94, 106 mg/kg bw/day (3 ♂) 41, 47, 54 mg/kg bw/day (3 ♀) Main study: 28, 56 and 112 mg/kg bw/day (6 ♂) 14, 28 and 56 mg/kg/bw day (6 ♀) 3 days Vehicle: corn oil Positive control: Cyclophosphamide Monohydrate (MN) (50 mg/kg bw/day) EMS (Comet assay) (200 mg/kg bw/day)	Genipin CAS No. 6902-77-8 Purity: 99.272%	Negative	1 (Reliable without restriction)	High/high	Documentation provided to EFSA No. 6

Abbreviations: B[a]P, benzo[a]pyrene; bw, body weight; CAS, Chemical Abstract Service; DFR, dose range finding; DMSO, dimethyl sulfoxide; EMS, ethyl methanesulfonate; HCD, historical control data; MN, micronucleus; NR, not reported; SCE, sister chromatid exchange.

ANNEX A

Exposure data and estimates

Table A1: Population groups considered for the exposure estimates of jagua (genipin-glycine) blue with DietEx
Table A2: Summary of total estimated exposure to jagua (genipin-glycine) blue from use as a food additive estimated with the DietEx tool for the proposed
Table A3: Main food categories contributing to exposure to jagua (genipin-glycine) blue estimated with the DietEx tool using the maximum level exposure