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SCIENTIFIC OPINION

Follow-up of the re-evaluation of silver (E 174) as a food additive (EFSA-Q-2023-00169)

EFSA Panel on Food Additives and Flavourings (FAF) | Monica Andreassen | Gabriele Aquilina | Maria Lourdes Bastos | Polly Boon | Laurence Castle | Biagio Fallico | Reginald FitzGerald | Maria Jose Frutos Fernandez | Bettina Grasl-Kraupp | Ursula Gundert-Remy | Rainer Gürtler | Marcin Andrzej Kurek | Henriqueta Louro | Patricia Morales | Sabina Passamonti | Agnes Oomen | Emanuela Corsini | Matthew Wright | Peter Furst | Eric Gaffet | Katrin Loeschner | Jan Mast | Anna Undas | Agnieszka Mech | Ana Maria Rincon | Laura Ruggeri | Camilla Smeraldi

Correspondence: fip@efsa.europa.eu

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

Abstract

Silver (E 174) is a food colour that was re-evaluated by the EFSA ANS Panel (2016). The ANS Panel concluded that the information available then, was insufficient to assess the safety of silver as food additive. The major issues included limited characterisation of silver E 174 (e.g. quantity of nanoparticles) and release of ionic silver. Following a European Commission call for further data to fill the data gap, the Panel on Food Additives and Flavourings (FAF) was requested to assess the safety of silver (E 174). One interested business operator (IBO) submitted limited data on particle size distribution and morphology, two genotoxicity studies and one subchronic study. The Panel concluded that the technical data submitted on physicochemical characterisation of all types of silver used as food additive E 174 were not adequate. As a result, the Panel was unable to propose changes to the EU specifications of E174 on particle size and morphology. As the additional information requested was not provided, the assessment was based solely on the submitted data. Nonetheless, given the data provided and silver insolubility in water, the Panel concluded that E174 requires risk assessment at the nanoscale following the EFSA Guidance on Risk assessment of nanomaterials to be applied in the food and feed chain, to complement the conventional risk assessment. The Panel considered that the genotoxicity data and sub-chronic toxicity data were inadequate. Consequently, the Panel could not conclude on the safety of the food additive silver E 174.

KEYWORDS

E 174, follow-up, food additive, food colourant, silver

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SUMMARY

The present opinion deals with the follow-up on issues that have been expressed in the conclusions and recommendations of the Scientific opinion on the re-evaluation of silver (E 174) as a food additive (EFSA ANS Panel, 2016).

Silver (E 174) is a food colour that was re-evaluated by the ANS Panel in 2016 (EFSA ANS Panel, 2016). EFSA ANS Panel (EFSA ANS Panel, 2016) concluded that the available data were insufficient to conclude on the safety of silver used as food additive E174. The major issues identified included the characterisation of silver E 174, particularly the quantity of nanoparticles and the release of ionic silver, as well as the lack of comparable information for the materials used in available toxicity studies, which hampered the determination of the relevance of available toxicological studies to the safety evaluation of silver as a food additive.

As a follow-up to that re-evaluation opinion, the EC issued a public call to gather data from IBOs that could reduce the uncertainties and the gaps in the dataset previously identified by the ANS Panel.

In response to the EC call for data, one interested buissnes operator (IBO) provided information on characterisation, manufacturing processes, particle size and morphology of silver used as a food additive (E 174).

According to the information provided to EFSA, E 174 is produced by two different processes: (i) 'atomisation process' to produce silver powder and (ii) 'leaves process' to produce either silver leaves or powder.

The IBO provided limited information on particle size of E 174. Only one qualitative SEM image was provided for E 174 produced by the 'atomisation process' and no data were provided for E 174 produced as leaves by the 'leaves process'. Incomplete quantitative information on particle size was provided for only two samples of E 174 produced as powder by the 'leaves process'. The Panel noted that the limited number of samples examined from a single manufacturing process may not be representative of all silver used as E 174 on the EU market. The average and median thickness of silver platelets were reported to be the same: 170 nm for one sample and 182 nm for the other sample. The fraction of particles (by number) with a thickness less than 100 nm was 0% and 3% for the two samples, respectively. The IBO provided a proposal on how to characterise E 174 in the EU specifications regarding particle size, which was not substantiated by the provided analytical data.

The Panel identified significant uncertainties in the data on particle size distribution and morphology provided by the IBO. Despite EFSA's request for additional information to clarify these uncertainties, the IBO did not provide clarifications. Consequently, the information provided by the IBO was considered insufficient to fully characterise the materials used as food additive E 174. Therefore, the Panel was unable to propose specifications for silver (E 174) in relation to the morphology and size of the particles, as requested in the Terms of Reference.

Considering the data provided on particle size distribution for silver (E 174) produced as powder in the 'leaves process' and that silver is insoluble in water, in line with the EFSA Guidance on Particle – Technical Requirements , the Panel considered that E174 requires risk assessment at the nanoscale following the EFSA Guidance on Nano – Risk Assessment , to complement the conventional risk assessment conducted according to the applicable sectoral guidance.

No data were provided on the release of silver ions from the food additive as requested in the EC call for data.

Due to lacking information and the remaining uncertainties about key properties of E 174 (e.g. such as particle size distribution, shape, composition and silver ion release) the Panel considered that conducting a literature search to identify additional evidence from the open literature to evaluate the safety of E 174, was not feasible. As a result, the current assessment was based solely on the data submitted in response to the EC call for data.

For the genotoxicity assessment, the IBO provided one in vitro and one in vivo study using the Prediscreen assay, which is not validated for regulatory risk assessment, nor recommended by EFSA or Organization for Economic Co-operation and Development (OECD) guidelines. The Panel found both studies not reliable, with inadequate sample preparation for nanoscale assessment, making the data of low relevance for the assessment of genotoxicity of E 174. Consequently, the Panel was of the view that the studies did not fulfil the requirements for genotoxicity testing according to EFSA relevant guidances.

The IBO submitted one dietary sub-chronic toxicity study with E 174, produced as powder by the 'leaves process', in mice. The Panel considered the study limited in assessing inflammatory changes in the limited number of organs examined. Additionally, insufficient information on silver E174 incorporation and aggregation/agglomeration in feed compared to commercial foods, the Panel considered that the relevance of the results of the sub-chronic toxicity study for assessing nano-sized particles/aggregates cannot be verified. Therefore, the Panel was of the view that the data obtained from the sub-chronic toxicity study, are not sufficient to conclude on the safety of the food additive.

Overall, the Panel considered the data submitted in response to the EC call for data insufficient. They lacked adequate physicochemical characterisation of all silver types used as E 174 on the EU market and did not include data on silver ion release. The genotoxicity data were inadequate to assess the genotoxic hazard, and the available toxicity data were insufficient to evaluate the safety of silver (E 174) under EFSA relevant guidance (EFSA ANS Panel, 2012; EFSA Scientific Committee, 2021a). Therefore, the Panel could not conclude on the safety of silver used as food additive E 174.

1 | INTRODUCTION

The present opinion deals with the follow-up on issues that have been expressed in the conclusions and recommendations of the Scientific opinion on the re-evaluation of silver (E 174) as a food additive (EFSA ANS Panel, 2016).

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

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 in Annex II to that Regulation, may be placed on the market and used in foods under the 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]

Silver (E 174) is authorised for use as a food additive in the Union. Since silver (E 174) was permitted in the Union before 20 January 2009, it belongs to the group of food additives which are subject to a new risk assessment by the European Food Safety Authority (EFSA), according to Commission Regulation (EU) No 257/2010,[3] and in line with the provisions of Regulation (EC) No 1333/2008.

EFSA completed the re-evaluation of silver (E 174) as a food additive and published a scientific opinion on 21 January 2016.[4] In that opinion, EFSA concluded that the information available was insufficient to assess the safety of silver as a food additive. EFSA also made recommendations concerning the specifications for E 174.

Consequently, the European Commission published on 6 March 2018 a call for data[5] requesting business operators to submit data addressing the conclusions and recommendations from the EFSA re-evaluation of the safety of silver (E 174) as a food additive. In particular, the call for data requested:

- Data on particle size and particle size distribution for E 174;
- Toxicological data: a toxicological database should be generated with the food additive silver (E 174), and in line with the tiered approach described in the EFSA's "Guidance for submission for food additive evaluations" (EFSA ANS Panel, 2012);
- Data on the release of silver ions from elemental silver in E 174.

In September 2021, a single business operator submitted data in reply to that call.

Terms of Reference

In accordance with Article 29(1)(a) of Regulation (EC) No 178/2002,[6] the European Commission requests the European Food Safety Authority (EFSA) to provide an updated scientific opinion on the safety of the food additive silver (E 174) and its specifications:

(i) Confirming that the analytical data provided by interested business operators adequately support the proposed amendment of the specifications of the food additive silver (E 174) including, but not limited to, additional parameters related to the particle size and particle size distribution.

(ii) Assessing the toxicity database in support of the safety of the proposed amendment to the specifications of the food additive silver (E 174). In particular, EFSA is requested to re-evaluate the database for the food additive silver (E 174) taking into account the data submitted by business operators in reply to the call for data issued by the European Commission, as well as any new relevant data retrieved from the published literature.

(iii) In line with the EFSA "Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles" (EFSA Scientific Committee, 2021a), EFSA should also consider whether the material, or a fraction of it, does require specific assessment of properties at the nanoscale.

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

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

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

[4]https://www.efsa.europa.eu/en/efsajournal/pub/4364.

[5]https://ec.europa.eu/food/system/files/2019-01/fs_food-improvement-agents_reeval_call_20180410_E174_data.pdf.

[6]Regulation (EC) no 178/2002 of the European Parliament and of the council of 28 January2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1.

1.2 | Summary of the EFSA Re-evaluation of silver E 174 as a food additive and other relevant assessments on silver

Silver (E 174) was re-evaluated by the EFSA ANS Panel (EFSA ANS Panel, 2016) which concluded that: 'the information available was insufficient to assess the safety of silver as food additive. The major issues included chemical identification and characterisation of silver E 174 (e.g. quantity of nanoparticles and release of ionic silver) and similar information on the material used in the available toxicity studies. Therefore, the Panel concluded that the relevance of the available toxicological studies to the safety evaluation of silver as a food additive E 174 could not be established'.

The ANS Panel highlighted the following data gaps and concerns with respect to silver (E 174) when used as a food additive:

- Data from toxicity studies on elemental silver or the food additive (E 174) were lacking.
- Data on particle size distribution of the food additive (E 174) were not available.
- There was evidence for the release of silver ions from elemental silver, which is of concern. However, the extent of any silver ion release, which depends on multiple factors such as pH and particle size, was unknown in the case of silver (E 174) when used as food additive.

The EFSA ANS Panel (2016) recommended that:

- The specifications for E 174 should include the mean particle size and particle size distribution (± SD), as well as the percentage (by number) of particles in the nanoscale (with at least one dimension below 100 nm), present in the powder form of silver (E 174) used as a food additive. The methodology applied should comply with the EFSA Guidance document (EFSA Scientific Committee, 2011), e.g. SEM or TEM.
- The Panel recommended that additional data in line with the current Guidance document on evaluation of food additives (EFSA ANS Panel, 2012) would be required.

1.3 | Information on other assessments on silver since the 2016 EFSA re-evaluation

Information from the 2021 EFSA safety assessment of silver nanoparticles in food contact materials

In 2021 the EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP) evaluated the safety of silver nanoparticles used at up to 0.025% w/w in non-polar plastics intended to come into contact with a variety of foods (EFSA CEP Panel, 2021). The silver nanoparticles were characterised by a size range of 1–100 nm with a mean diameter of approximately 15 nm and with 99% of the particles below 20 nm in size. When incorporated into plastics, the nanoparticles remain in particulate form, although some aggregation and agglomeration were observed. Data from migration studies and abrasion tests along with theoretical considerations based on migration modelling, indicated that the silver nanoparticles remain embedded in the plastic, do not migrate and resist release by abrasion. A low-level of migration (up to 6 μg/kg food) of silver in its soluble ionic form was detected, well below the 50 μg/kg food group restriction for migration of silver (EFSA AFC Panel, 2004) and the estimated exposure was below the ADI of 0.9 μg silver ions /kg bw per day (ECHA and EFSA, 2020). The CEP Panel concluded that using silver nanoparticles (up to 0.025% w/w) in specific plastics intended for food contact poses no safety concern.

Information from the 2022 ECHA RAC opinion on CLH classification of silver

In 2022, the Risk Assessment Committee (RAC) of the European Chemicals Agency (ECHA) published its opinion on the classification of silver (CAS 7440-22-4) (ECHA RAC, 2022). The RAC opinion addressed silver in different forms described as: (i) bulk form with particles with size > 100 nm including: (i) 'massive form' (particle with size ≥ 1 mm diameter) and silver 'powder' (particles with size > 100 nm and < 1 mm) and (ii) 'nano silver' forms with particles with size > 1 nm and ≤ 100 nm. No further details in relation to the shape of the silver particles were provided in the RAC opinion.

The RAC opinion stated that 'data from studies using silver nanoparticles supporting classification for human health hazards should also be used to represent bulk forms of elemental silver such as massive and silver powder (including micron-sized silver dust). For practical reasons, silver nanoparticles were considered together, even though at an individual level, physicochemical characteristics such as size, shape, surface charge, surface functionalisation or core composition may be different and may influence their interactions with biological systems and affect their uptake, toxicokinetics and toxicodynamics'. Consequently, no distinction was made between different forms of silver in the nanoscale in the RAC assessment. The RAC did not support using read-across data from soluble silver salts or other silver compounds to metallic silver due to significant differences in their physicochemical properties and bioavailability. The RAC emphasised the need for hazard assessment that rely on data specific to the form of silver under consideration, rather than assuming equivalence between different silver-containing substances.[7]

[7]'Silver compounds (substances other than elemental silver, with their own EC and CAS numbers) that have the potential to release silver ions (Ag^+) into biological systems; these include (i) silver salts and (ii) a variety of low solubility silver compounds that act as slow-release matrices or ion exchangers (e.g. silver zinc zeolite)' (ECHA RAC, 2022).

Based on the provided data RAC classified silver in bulk form (massive and powder) and nano form as potentially causing damage to the nervous system (STOT RE 2; H373 (nervous system)) and suspected of impairing fertility (Repr. 2; H361f).

Information from the 2024 EC SCCS safety assessment on the use of silver in cosmetics products

The EC Scientific Committee on Consumer Safety (SCCS) evaluated silver powder used in cosmetics products which is highly porous (85%–90%; specific surface area up to 5 m^2/g) (SCCS, 2024). In its opinion it was stated that 'SEM imaging revealed that the substructures (not existing as individual entities but as a non-separable part of larger individual unbound units) were approximately spherical'. The number-based 'substructure-particle'[8] size of the evaluated substance ranged between 42.2 and 320 nm. The mean (±SD) measured 'substructure-particle' size was 122.4 ± 37.7 nm (SD: 84.7–160.1 nm). Further characterisation of the number-based 'substructure-particle' size distribution by SEM revealed specific percentiles: D10 measured at 80.5 nm, D50 at 116.2 nm and D90 at 172.8 nm.

As it could not be determined whether or not the detected amounts of silver in the dermal penetration study relate to particles or ions, the SCCS assumed that the particles dissolve fully to release ions. Dermal absorption was estimated at 2.14%, based on an in vitro study and the absorbed silver was assumed to exist as ions for the safety assessments.

The SCCS based its evaluation of systemic toxicity on exposure to silver ions, excluding studies on silver nanoparticles due to their different physicochemical characteristics and potential toxicological profile compared to the silver under assessment.

The SCCS concluded that silver, used in cosmetic products, is not irritating to the skin or eyes and presents a negligible risk for sensitisation. A no observed adverse effect level (NOAEL) of 0.0045 mg of silver ions equivalence /kg bw per day was identified based on pigmentation effects of internal organs (liver, kidneys, pancreas, stomach and lymph nodes choroid plexus) in a chronic toxicity study involving crystalline powder of silver zinc zeolite, corrected for oral bioavailability (Takizawa, 1992). While the SCCS agreed with RAC (RAC, 2022) that silver should be classified as a Category 2 reproductive toxicant for adverse effects on sexual function and fertility, the data from the studies with the nano-forms were not used in the SCCS assessment. 'The SCCS concurs with ECHA – RAC (2022) that a classification for mutagenicity is not warranted '. Furthermore, the SCCS referred to its opinion on packaging material releasing silver (SCCS, 2016), where SCCS concluded that genotoxicity/mutagenicity data submitted were inconclusive. The SCCS considered that the main mechanism of genotoxicity of silver ions is via reactive oxygen species (ROS) production, which is an indirect process dependent on concentration levels and since the concentrations of silver ions present in cosmetic products are low, the SCCS had no concern regarding human risk from the use of silver under assessment in cosmetics products. For carcinogenicity, the SCCS concurred with the RAC opinion (RAC, 2022) that no classification for carcinogenicity can be proposed due to inconclusive data. Furthermore, the SCCS stated in its opinion on silver zinc zeolite (SCCS, 2023) that it agrees with the ECHA Biocidal Products Committee Opinion (ECHA BPC, 2021) that silver zinc zeolite is not likely to be carcinogenic. Based on these findings, considering the NOAEL of 0.0045 mg/kg bw per day (expressed as silver ions equivalents), the SCCS calculated systemic exposure and a bioavailability factor of 0.01% for oral products. The SCCS concluded that silver is not safe at concentrations of 0.2% in rinse-off and 0.3% in leave-on products. Its use in eye shadow, lip balm, toothpaste and mouthwash at specified levels was considered safe.

The Panel noted that the silver assessed by the SCCS (SCCS, 2024) for use in cosmetics differs significantly from the silver used as E 174 as described in Section 3.2.1.

The Panel further noted that currently, silver is under evaluation by ECHA as a potential endocrine disruptor.[9]

Furthermore, there is an ongoing project on the 'Integration of new approach methodologies results in chemical risk assessments: case studies addressing nanoscale considerations'[10] funded by EFSA in which 'conventional silver with a nanoscaled fraction authorised as food additive (E 174)'[11] is considered as a case study'.

2 | DATA AND METHODOLOGIES

2.1 | Data

The Panel based its assessment on the information submitted following the European Commission call for data.[12] One interested business operator (IBO), Consortium of Producers of Edible Gold and Silver of Europe, submitted information (Documentation provided to EFSA No. 1).

[8]The SCCS considered that the substructure-particle corresponds to constituent particle.

[9]https://echa.europa.eu/it/substance-information/-/substanceinfo/100.028.301 (accessed on 17.12.2024).

[10]https://www.efsa.europa.eu/en/news/nanotechnology-promoting-uses-new-assessment-methods.

[11]https://www.iss.it/en/nams4nano-lot2.

[12]https://food.ec.europa.eu/document/download/fb9f972e-4767-4e7e-91c3-9e7fc9ff62d1_en?filename=fs_food-improvement-agents_reeval_call_20180410_e174_data.pdf.

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) has also been followed.

Terms and definitions related to nanomaterials used in this document are as described by the European Commission's Joint Research Centre (Rauscher et al., 2023).

The Panel noted that in the data submitted, the IBO used various terms to describe the morphology of silver particles (e.g. plates, foils, leaves, sheets). According to ECHA Guidance (ECHA, 2022), particles with one external dimension significantly smaller than the other two external dimensions, where this smaller dimension represents the thickness of the particle, fall under the shape category 'platelets'. The Panel applied the ECHA shape terminology to describe the morphology of E 174 particles in this assessment while the reported data provided by the IBO includes the terminology used by the IBO itself.

The data provided by the IBO in response to the call for data show that the constituent particles of silver used as E 174 are in the nano size range with median minimal external dimension values from 170 to 182 nm (see Section 3.2.1 of this Opinion). Silver is insoluble in water (ECHA, 2022; SCCS, 2024) (see Section 1.2). Therefore, in line with the EFSA Guidance on Particles – Technical Requirements (EFSA Scientific Committee, 2021a), E 174 requires a risk assessment at the nanoscale following the EFSA Guidance on Nano – Risk Assessment (EFSA Scientific Committee, 2021b), to complement the conventional risk assessment conducted according to the applicable sectoral guidance.

Due to insufficient information and uncertainties about key properties of silver (E 174) as a food additive (see Section 3.3), such as particle size, shape, composition and silver ion release, the Panel deemed it unfeasible to conduct a search of the published literature to identify additional evidence to evaluate the safety of E 174, as requested in the Term of Reference (see Section 1.1). Consequently, the Panel performed the assessment relying solely on data submitted by the IBO in response to the EC call for data.

3 | ASSESSMENT

3.1 | Identity and specifications

Specifications for silver (E 174) have been defined in Commission Regulation (EU) No 231/2012 on specifications for food additives (Table 1).

TABLE 1 Specifications for silver (E 174) according to Commission Regulation (EU) No 231/2012.

	Commission Regulation (EU) No 231/2012
Assay	Content not less than 99.5% Ag
EINECS	231-131-3
Description	Silver-coloured powder or tiny sheets
Identification	No information
Purity	No information

3.2 | Technical data submitted

The following was requested in the European Commission call for data[13]:

- Information on particle size and particle size distribution for the food additive silver (E 174) supported by analytical data, in line with the draft 'EFSA guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health'.
- Detailed and comprehensive proposed specifications for the characterisation of the fraction of nanoparticles present in the food additive silver (E 174).

One IBO, a consortium of Producers of Edible Gold and Silver of Europe, provided information in response to the call for data.

[13]https://food.ec.europa.eu/document/download/fb9f972e-4767-4e7e-91c3-9e7fc9ff62d1_en?filename=fs_food-improvement-agents_reeval_call_20180410_e174_data.pdf.

3.2.1 | Characterisation of silver used as E 174

According to information submitted by the IBO, silver used as a food additive is described as 'a metallic material in the shape of small sheets and powders'. The IBO further reported that the composition of E 174 contains silver not less than 99.5% (w/w), and up to 0.5% (w/w) of gold and/or copper (Documentation provided to EFSA No. 1).

3.2.2 | Manufacturing process

The IBO reported that silver used as E 174 is produced by two different processes (see Figure 1) described as the:

- '**Atomisation process**' to produce **silver powder**. In this process the metal (silver) is melted and atomised into drops. A rolling process is then used to make the drops thinner and to obtain only powders consisting of particles in the form of plates ('drops crushed by the rolling'). The IBO stated that the particles are 'homogeneous and non-porous' (Documentation provided to EFSA No. 1).
- '**Leaves process**' to produce either **silver leaves** or **powder**. In this process, after an initial melting and casting, several mechanical processes of deformation i.e. rolling and beating, result in silver in the form of very thin leaves. The final steps of the manufacturing process i.e. cutting, shredding or grinding, result in either sheets or powders. The IBO stated that 'these particles are homogeneous, non-porous, non-agglomerated and non-aggregated' (Documentation provided to EFSA No. 1).

The IBO further stated that in both manufacturing processes, the particles typically have the characteristic appearance of silver leaf fragments, with two relatively large and irregular dimensions and one small and regular dimension representing the thickness of the leaf.

3.2.3 | Particle size distribution and morphology

The IBO provided information on particle size and morphology of E 174 in the form of **powders** produced by the 'atomisation process' and 'leaves process'. No such information on E 174 produced as **leaves** by the 'leaves process' were provided (see Figure 1), (Documentation provided to EFSA No. 1).

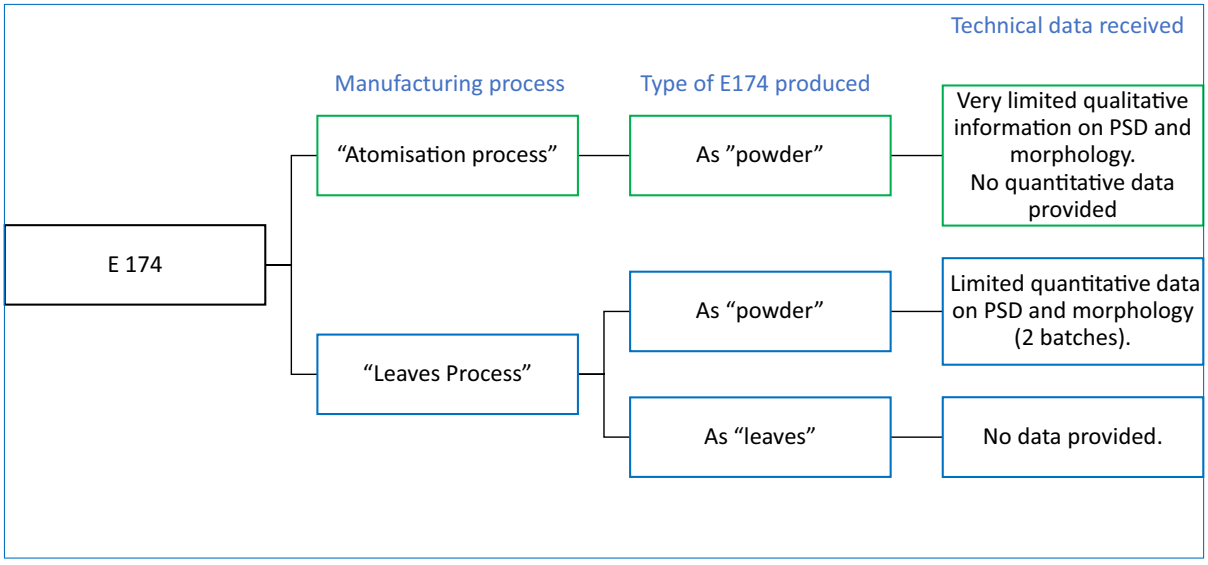

FIGURE 1 Overview of technical data on particle size and morphology received for E 174 assembled by the Panel.

Silver E 174, atomisation process

For silver E 174 produced as powder by the atomisation process, only a qualitative description of one sample based on one SEM image was provided and it is presented here as Figure 2. The method of analysis was only briefly described. The Panel noted that quantitative data on particle size distribution (lateral dimensions and thickness) were not provided. The image showed plates with micrometric lateral sizes. The IBO reported that a few 'plates' or 'parts of plates' with submicrometric lateral sizes were observed, but no particles with lateral dimensions below 100 nm were detected. The IBO stated that due to the wide range of 'plates' sizes in the analysed powder, it was not possible to establish a number-based particle size distribution histogram using SEM analysis.

The IBO complemented the SEM analysis with energy dispersive X-ray spectroscopy (EDX) analysis, confirming that the particles visualised in the electron micrograph contained silver (Documentation provided to EFSA No.1).

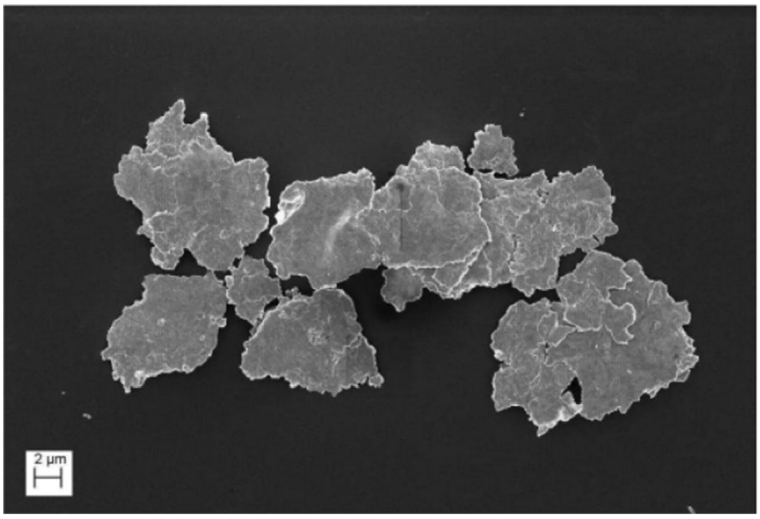

FIGURE 2 SEM image of E 174 produced as powder by the 'atomisation process'.

The Panel noted from the SEM image provided, that the material had rough edges. The Panel considered that the distinctly separate submicrometric plates could be agglomerates and/or aggregates of smaller particles but that this could not be unequivocally established based on the single image provided. The magnification (resolution) of the image (see Figure 2) does not allow visualisation of constituent particles in the nano range, as indicated as a requirement in the EFSA Guidance (EFSA Scientific Committee, 2021a; EFSA Scientific Committee, 2021b).

Additionally, for this sample of silver (E 174), the IBO provided information on the volume specific surface area (VSSA) measured by the BET method. The Panel noted that the BET method is not considered suitable to investigate for the presence of nano-sized particles as this method does not allow to accurately measure the size of the constituent particles as required by the Guidance on Particle-TR (EFSA Scientific Committee, 2021a) and Guidance on nano RA (EFSA Scientific Committee, 2018; EFSA Scientific Committee, 2021b) and is prone to bias for polydisperse materials (Mech, Rauscher, et al., 2020; Mech, Wohlleben, et al., 2020; Rauscher et al., 2019, 2023).

Silver E 174, leaves process

The IBO provided data on particle size and particle morphology based on SEM analysis for two samples of silver E 174 produced as powder by the 'leaves process' and considered them as 'the case of the tiniest particles' produced. The method of analysis was described, quantitative data on particle size (determined by measuring Feret minimal (thickness) dimension) were provided and statistical analyses performed (see Table 2) (Documentation provided to EFSA No. 1).

Based on the SEM images, the IBO reported that fragments of 'foils'[14] had lateral dimensions ranging from a few micrometres to several hundred micrometres, with no particle having one or two lateral dimensions below 100 nm (Documentation provided to EFSA No. 1).

Thickness measurements of 100 different fragments of 'foils' (see Figure 3) were performed on images from both samples of silver powder. Thickness measurements were performed on three distinct points of foil edges. The parameters determined from the average thickness measurements of each fragment of foil are shown in Table 2.

[14]According to ECHA Guidance Particles with one external dimension significantly smaller than the other two external dimensions and for which smaller external dimension is the thickness of the particle falls under the shape category 'platelets'. Examples of shapes covered under this category are discs, plates, etc. For the sake of harmonisation and clarity the Panel applied the ECHA shape terminology to describe the morphology of E 174 particles in this assessment. The reported data provided by the IBO includes the terminology used by the IBO itself.

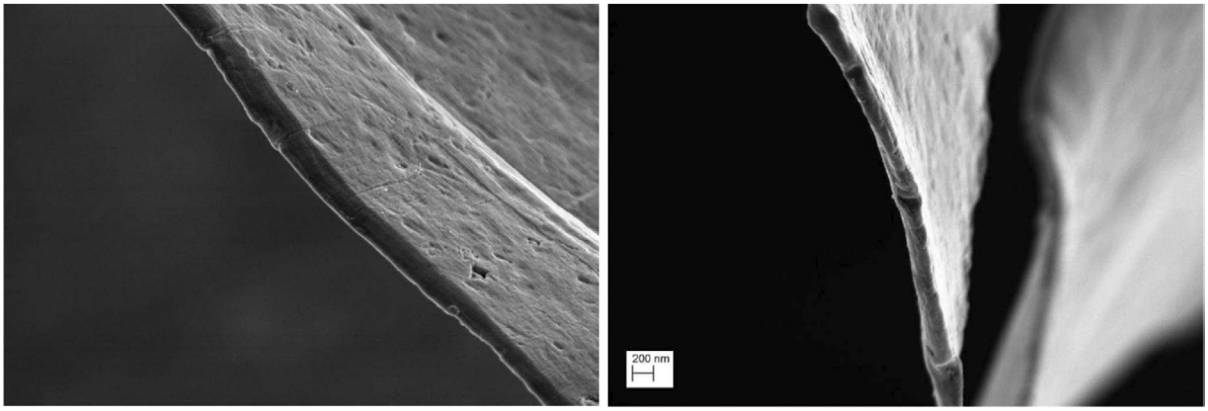

FIGURE 3 Example of SEM image provided for sample 1 of E 174 produced as powder by the 'leaves process'.

TABLE 2 Particle size measured (averaged thickness of foils) by SEM as reported by the IBO (Documentation provided to EFSA No. 1).[a]

Silver powder lot	Sample 1	Sample 2
Average size	170.2 nm ± 20.6 nm	182.4 nm ± 9.7 nm
Distribution width (standard deviation (SD))	44.3 nm	63.5 nm
Median size	170.2 nm	182.4 nm
Mode[b]	170.2 nm	182.4 nm
Fraction of fragments (by number) with thickness < 100 nm	3%	0%

[a]The Panel assumed that the data provided in the table correspond to the normal distribution fit of the averaged thickness of the foils.
[b]The mode of the distribution is described by the IBO as the average size of the most common class (Documentation provided to EFSA No. 1).

The Panel noted that the identity of the variation (i.e. ± 20.6 nm, 9.7 nm) reported for the average size was not explained. The Panel assumed that it represents the standard error of statistics.

The Panel noted that the silver particles obtained in the form of a powder by the leaves process exhibit a platelet[14] morphology with one small regular dimension (the particle's thickness) and two large irregular dimensions. The IBO reported that these particles are homogeneous, non-aggregated and non-porous. The Panel noted that the IBO did not explain which feature of the particles the term 'homogenous' refers to; nevertheless the Panel assumed that the IBO means that the particles exhibit similar morphology. The Panel further noted that the material shows smoother edges in comparison to the material obtained by the atomisation process. The Panel noted that the minimum and maximum observed platelets thicknesses across all measurements for both samples were not provided (Documentation provided to EFSA No. 1).

The IBO also provided results of an Atomic Force Microscopy (AFM) analysis performed on one sample of E 174 produced as powder by the 'leaves process', aiming to determine the thickness of the silver foils. All measured thickness values were reported above 100 nm (Documentation provided to EFSA No. 1).

The Panel noted that based on the data provided by the IBO (Median (D50), 170.2–182.4 nm) more than 10% of particles of silver used as E 174 produced as powder by the 'leaves process' has at least one dimension smaller than 250 nm. Therefore, considering this feature and following the EFSA Guidance on Particle-TR (EFSA Scientific Committee, 2021a), the Panel is of the view that the silver used as E 174 contains small particles including nanoparticles.

3.2.4 | Proposed specifications by the IBO

The IBO proposed the following specifications for the characterisation of the fraction of nanoparticles present in the food additive silver (E 174) (Documentation provided to EFSA No. 1):

- '10% maximum of particles (by number) with one dimension below 100 nm';
- '1% of particles (by number) with one dimension below 50 nm'.

For the silver produced by the 'leaves process' the IBO provided information on the average thickness calculation of the silver platelets estimated from the production parameters based on 'the weight of the leaf' expressed as 'thickness =weight/surface/density (10.5)' for the range of samples weighing from 10 to 35 g/1000 leaves (Documentation provided to EFSA No. 1).

The IBO further stated that 'according to the ranges of thickness of silver leaves produced by manufacturers and the results of analysis performed on several batches of E 174' produced by the 'leaves process', the estimated maximum level

of particles with one dimension below 100 nm is 10% (in number) for the batches of E 174 containing the 'thinnest silver leaves' (10 grams in weight, calculated to be 149 nm) (Documentation provided to EFSA No. 1).

However, the Panel noted that no explanation on how the parameters used in the thickness calculations were determined nor were any analytical data presented to support this estimation. The Panel further observed that the SEM quantitative analysis was performed on the two samples of '12 grams in weight' with a measured average platelet thickness of 170 nm in one sample and 182 nm in the other sample and not on the 'thinner' sample of '10 grams in weight'.

Additionally, the Panel noted that the specifications provided by the IBO were based only on the data for silver produced by the 'leaves process' and not for E 174 produced by the atomisation process.

The Panel noted that limited justification (analytical data for two samples) for the proposed maximum content of 10% (by number) of particles below 100 nm in silver was provided. The Panel also considered that the data did not support the proposal of 1% (by number) of particles with one dimension below 50 nm. Furthermore, the Panel noted that the IBO did not provide a proposal for the description of the morphology to be included in the EU specifications for E 174.

3.3 | Assessment of the technical data submitted

The IBO stated that silver used as the food additive E 174 is produced by two manufacturing processes: the 'atomisation process' yielding E 174 in powder form, and the 'leaves process' resulting in silver in the form of leaves or powders (Documentation provided to EFSA No. 1).

Composition

The IBO stated that E 174 comprises not less than 99.5% silver (w/w) and up to 0.5% (w/w) of gold and/or copper. The Panel noted that the reported purity (not less than 99.5%) meets the limit indicated for E 174 in the EU specifications set in the Commission Regulation (EU) No 231/2012. However, the presence of gold and/or copper up to 0.5% (w/w) in the food additive is not indicated in the EU specifications for E 174. The Panel further noted that the origin of gold and/or copper in E 174 is unclear. The IBO did not respond to EFSA's additional request for clarifications on this aspect.

Morphology and particle size

The IBO provided information on the morphology and particle size of silver (E 174) produced as powder by the 'atomisation process' and the 'leaves process'. In both cases, SEM images showed that the particles are in the form of platelets, with one dimension (the thickness of the platelet) being significantly smaller than the two other (lateral) dimensions. The IBO reported that no silver foils with lateral dimensions below 100 nm were observed. Quantitative information on particle size was provided for two samples of E 174 produced as powder by the 'leaves process' only. The average thickness was reported to be the same as the median thickness and was 170 nm for one sample and 182 nm for another sample. The fraction of particles (by number) with a thickness less than 100 nm was 0 and 3% for the two samples (Documentation provided to EFSA No. 1).

The IBO proposed an amendment of the EU specifications of E 174 to define the food additive as containing a maximum of 10% of particles (by number) with one dimension below 100 nm and of 1% of particles (by number) with one dimension below 50 nm (Documentation provided to EFSA No. 1). The Panel noted that these thresholds and maximum contents of particles in number are not substantiated by the analytical data submitted. Furthermore, the IBO did not provide any proposal for the description of the morphology to be included in the EU Specifications for E 174.

Based on the information provided by the IBO that the variation of the smallest dimension of the particles is related to the variability of the manufacturing process and to the different production parameters of the silver leaves produced by the manufacturers, the Panel noted that the limited number of examined samples produced by the 'leaves process' might not be representative of all types of silver used as E 174 available on the market.

Moreover, the Panel noted that:

- The IBO did not provide quantitative information on the lateral dimensions of the silver platelets produced in the leaves process. Small particles with lateral dimensions in the nano range were suspected based on SEM images. However, the low magnification/resolution of these images does not allow for a thorough assessment. The IBO did not respond to EFSA's additional request for clarifications on this aspect.
- The IBO estimated that in E 174 the maximum fraction of particles with one dimension below 100 nm is 10% (by number). However, information from the literature (De Vos et al., 2020; Waegeneers et al., 2019) indicated that nanoparticles are released from confectionary products containing E 174 when these were introduced in water. In these conditions, in all samples and independent of the choice of analytical technique (TEM or SP-ICP-MS), the nano-sized particles represented more than 97% (by number) with a median size (D50) of 11 nm (TEM) and with spheroidal morphology, even though the largest fraction (99.45%) of the silver mass was present as flakes with very rough edges (De Vos et al., 2020). Therefore, as reported in these studies the percentage by number of the nanoparticles from E 174 could be higher than that reported by the IBO. Clarification on this aspect was requested, but the IBO did not respond to EFSA's request.

- For the silver produced as powder by the 'atomisation process' the IBO did not provide any quantitative data and only one SEM image with a magnification (resolution) which does not allow visualisation of constituent particles in the nano range and does not allow to estimate the thickness of the particles. Clarifications on that aspect were requested, but the IBO did not respond to EFSA's request. Therefore, the Panel cannot exclude that the E 174 obtained by the atomisation process contains a much higher percentage than 10% (by number) of the particles with one dimension below 100 nm, as proposed in the specifications by the IBO.
- The submitted data do not provide information on the minimal external dimension (lowest measured thickness) of the silver platelets produced by both processes. Clarification on this aspect was requested, but the IBO did not respond to EFSA's request.
- It is not clear whether silver used as food additive is coated or functionalised. Clarification on that aspect was requested, but the IBO did not respond to EFSA's request.

Overall conclusion on technical data

Taking into account the data submitted in response to the EC call for data for the follow-up of the re-evaluation of silver (E 174), the Panel considered that the data provided by the IBO on the characterisation of silver used as E 174 were insufficient to characterise the materials used as this food additive. The Panel was therefore unable to propose changes to the current EU specifications for silver (E 174) in relation to the morphology and particles size, as requested in the Terms of Reference (see Section 1.1).

3.4 | Biological and toxicological data submitted

The following was requested in the call for data[15]:

- A toxicological database generated with the food additive silver (E 174), and in line with the tiered approach described in the EFSA's current guidance for submission for food additive evaluations (EFSA ANS Panel, 2012).

In response to the call for data, the IBO provided only data on genotoxicity and sub-chronic toxicity, described below.

3.4.1 | Genotoxicity

No genotoxicity studies as recommended in the EFSA Guidance (EFSA ANS Panel, 2012) were submitted by the IBO in response to the EC call for data.

The IBO provided an in vitro and an in vivo study using the Prediscreen assay. This assay detects changes in histone H2AX phosphorylation (γH2AX) status and expression levels of phospho-H3 protein (p-H3) as markers of clastogenic and aneugenic events, respectively.

The Panel noted that the Prediscreen assay, both in vitro or in vivo, is not validated for regulatory risk assessment; is not a test recommended in the current EFSA guidance documents (EFSA Scientific Committee, 2011, 2017), and it is not included in the OECD testing guidelines recommended for the assessment of potential genotoxicity (OECD, 2017). The Panel also noted that sample preparation in the in vitro and the in vivo Prediscreen studies was not adequate for the assessment at the na-noscale as described in the EFSA Guidance (EFSA Scientific Committee, 2021b).

3.4.1.1 | *In vitro Prediscreen assay*

The IBO provided a report on the genotoxicity of E 174 in vitro using a Prediscreen assay. (Documentation provided to EFSA No. 2).

The PrediScreen assay was performed in three human cell lines, namely HepG2 (liver), LS-174T (colon) and ACHN (kidney) (for details see Appendix A). The cells were exposed for 24 h to: (i) E 174 (powder) as either un-sonicated or sonicated samples (stock solution 2 mg/mL in water sonicated for 10 min) at 0, 20, 40, 80 and 160 µg/mL or (ii) to silver nanoparticles[16] (AgNPs, material for comparison) in a sonicated sample (stock solution 2 mg/mL in water sonicated 10 min) at 0, 80 and 160 µg/mL. The Panel noted that insufficient information on sample preparation, including sonication details, were provided. In addition, the Panel noted that no justification on the selection of the top concentration (160 µg/mL) was given. Benzo[α] pyrene, etoposide or nocodazole were used as positive controls for γH2AX and p-H3, respectively.

No cytotoxicity was observed at any of the tested concentrations for both tested materials in all three cell lines. The exposure to 80 and 160 µg/mL of the sonicated E 174 induced a concentration-dependent and statistically significant increase ($p < 0.01$) in γH2AX in the HepG2 cells, but not in LS-174T and ACHN cells, as compared to the negative control.

[15] https://food.ec.europa.eu/document/download/fb9f972e-4767-4e7e-91c3-9e7fc9ff62d1_en?filename=fs_food-improvement-agents_reeval_call_20180410_e174_data.pdf.

[16] Silver nanoparticles SkySpring Nanomaterials, Inc. Houston USA.

No effect of sonicated E 174 was observed on p-H3 for any of the four concentrations tested, in any cell lines. No effect of non-sonicated E 174 was seen on γH2AX and p-H3, for any of the four concentrations tested, in any cell lines. Sonicated AgNPs induced a statistically significant increase in γH2AX, but no change in p-H3 levels, in HepG2 cells at the highest concentration tested only (160 μg/mL).

An oxidative stress test was carried out with sonicated E 174 and N-acetylcysteine as an antioxidant, and in parallel with buthionine sulfoximine as a pro-oxidant to test the hypothesis that the γH2AX induction observed in treated HepG2 cells was associated with an increase in oxidative stress. The study author reported that sonicated E 174 induced a statistically significant increase ($p < 0.01$) in oxidative stress at 160 μg/mL after 4 h in vitro exposure in HepG2 cells, and a statistically significant ($p < 0.05$) and concentration-dependent increase at 80 and 160 μg/mL after 24 h of exposure.

The study author concluded that sonicated E 174 induced a clastogenic effect with a statistically significant and concentration-dependent increase in the induction of γH2AX phosphorylation at 80 and 160 μg/mL after 24 h exposure without cytotoxic effect in the HepG2 cells. The effect was not apparent in the LS-174T cells and in the ACHN cell line, suggesting that the E 174 had in HepG2 cells a clastogenic mode of action (Documentation provided to EFSA No. 2).

Considering that the study was conducted according to a method which is not validated (no Guideline available and not a GLP study), it was scored as not reliable according to EFSA Harmonised approach for reporting reliability and relevance of genotoxicity studies (EFSA, 2023). In addition, in the EFSA technical report (EFSA, 2023), the γH2AX test is considered 'a test with lesser relevance that can only be used as supporting information for hazard identification'.

3.4.1.2 | *In vivo Prediscreen assay*

The IBO submitted data from a PrediScreen assay performed on colon (segment of intestinal tissue) and liver (right median lobe) tissue of male and female C57BL/6J mice (Documentation provided to EFSA No. 3) from a repeated-dose 90-day oral toxicity study (see Section 3.4.2). Further details on the description of the study can be found in Appendix A.

Four groups of mice (10 mice/sex/group) were given E 174 (powder) (0, 1, 10, 100 μg/kg bw per day) or AgNPs[17] (100 μg/kg bw per day) in the diet for 90 days (see chapter 3.4.2 for considerations on feed preparation) (Documentation provided to EFSA No. 4).

The study authors justified the selection of the doses tested based on the human dietary exposure to E 174 estimated by the EFSA ANS Panel (2016).

The results showed that E 174 induced a statistically significant increase ($p < 0.01$) in phosphorylation of the biomarker H2AX (γH2AX) in the colon of female mice at all tested doses (1, 10 and 100 μg/kg bw per day) and in male mice at 10 and 100 μg/kg bw per day. In females, the effect was deemed to be dose-dependent, while in males the increase was not dose-dependent. Similarly, AgNPs induced a statistically significant increase of γH2AX at the single dose tested of 100 μg/kg bw per day ($p < 0.01$) in colons of both males and females. Neither E 174 nor AgNPs induced an effect in the biomarker H3 (p-H3) on colon and liver samples in either male or female mice.

The study author concluded that these findings suggest a potential clastogenic effect of E 174 and AgNPs on the colon of C57BL/6J mice, as evidenced by γH2AX induction.

The Panel noted that the IBO provided a summary of a BMD analysis of the response for γH2AX obtained in the colon of female and male mice, although the BMD report was not submitted.

Considering that the study was conducted according to a method which is not validated (no Guideline available and not a GLP study), it was scored as not reliable according to EFSA technical report (EFSA, 2023). In addition, in the EFSA technical report (EFSA, 2023), the γH2AX test is considered 'a test with lesser relevance that can only be used as supporting information for hazard identification'.

3.4.1.3 | *Overall summary of genotoxicity data*

The Panel considered that the data presented by the IBO are of low relevance for the assessment of genotoxicity of E 174. The genotoxicity studies on E 174 provided by the IBO in response to the EC call for data do not fulfil the requirements for genotoxicity testing specified in the EFSA ANS Panel guidance (EFSA ANS Panel, 2012). In view of the presence of small particles including nanoparticles in E 174, the genotoxicity data should have been generated taking into account the specific considerations applicable to this case and described in the EFSA Guidance documents on the risk assessment of nanomaterials (EFSA Scientific Committee et al., 2018, 2021b).

3.4.2 | Sub-chronic toxicity

The IBO submitted a sub-chronic oral toxicity study in mice (Documentation provided to EFSA No. 4).

Five-week-old male and female C57BL/6J mice (10/sex/ group) were given in the diet, for 90 days, E 174 (powder, 'leaves process') (0, 1, 10 or 100 μg/kg bw per day) or particulate silver nanoparticles (AgNPs) (0 or 100 μg/kg bw per day) for comparison. More information on the characterisation of the tested materials is given in Appendix B. The study authors justified

[17] Silver nanoparticles 50 nm in diameter, SkySpring Nanomaterials, Inc. Houston USA.

the selection of the doses tested based on the human dietary exposure to E 174 estimated by the EFSA ANS Panel (EFSA ANS Panel, 2016).

The feed was prepared once, by admixture and pellet formulation by compaction and kept in appropriate storage conditions for the duration of the study. The test items E 174 and AgNPs were added to the animal feed in the form of a powder. No further information was provided on the preparation of the feed with E 174 or AgNPs and the degree of aggregation/agglomeration of silver particles therein. Furthermore, no comparison to the aggregation/agglomeration of E 174 in feed to the aggregation/agglomeration of E 174 as present in marketed food was available. The Panel noted that also no information on the homogeneity of E 174 and AgNPs in the feed was provided.

The test items were well tolerated, no clinical signs were observed throughout the study and no morphological (macroscopic) abnormalities were observed on the day of sacrifice in the abdominal and pelvic organs. There were no deaths during the study and no significant differences in food intake and in body weight (detailed summary of the study is provided in the Appendix B).

All animals at the end of the study were examined for changes in gut microbiota (based on stool analysis); faecal lipocalin-2 levels; ileum permeability and selected colonic mucosal cytokine expression. However, particle analyses (jejunum, peyer's patches, colon, liver and spleen) and inflammatory scores (small intestine, colon, liver and spleen) in haematoxylin and eosin (H&E) stained tissue sections were limited to three animals per sex (data from all 6 animals were pooled) in the control and high dose E 174 and AgNPs groups.

Some changes in gut microbiota metabolic activity (based on stool analysis) were observed. However, a clear understanding of the significance of changes in structure and functionality of the gut microbiota in laboratory animals and their relevance with respect to human health is not yet elucidated; further research is ongoing and at present the Panel is not in a position to make any conclusion on this issue.

In the jejunum, particles were only rarely seen in Peyer's patches in both E 174- and AgNPs-treated mice. The presence of silver was confirmed by TEM-EDX in both E 174- and AgNPs- treated mice but there were no detectable changes in ileum permeability and no changes in inflammatory scores in small intestine H&E-stained tissue sections in either E 174- or AgNPs-treated mice.

In the colon, particles were rarely seen in E 174-treated mice but present in AgNPs-treated mice. However, in all the particles examined in colon tissue by SEM–EDX, silver was not detected. There were no changes in inflammatory pathology scores in the limited H&E-stained tissue sections examined from E 174-treated mice but an increase in score in AgNPs-treated mice.

The expression and production of pro- and anti-inflammatory cytokines by the colonic mucosa were evaluated using qPCR (RNA assay) and ELISA (protein assay), respectively. Limited information was provided on how the colonic mucosa was prepared for the tests.

Overall, in females exposed to E 174, a decrease in both pro- and anti-inflammatory cytokines were observed. While in males, the anti-inflammatory cytokine IL-10 could selectively inhibit IL-1β expression without affecting tumour necrosis factor-α (TNF-α) production, which levels were increased in E 174 treated animals at all doses.

In AgNPs-treated animals, a suppressive effect on the immune profile in the intestine of both male and female mice was observed. This conclusion was based on the reduction of both pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-10) cytokines. The discrepancy between intestinal inflammatory scores in H&E-stained tissue sections from AgNPs-treated animals and reduced cytokine levels in colon tissue could be explained by the localised nature of inflammation, timing, cytokine transport inefficiencies or degradation processes.

Regarding faecal Lcn-2 levels, no changes were observed in both AgNPs- and E 174-treated male mice. In females, a slight but not statistically significant increase in faecal Lcn-2 levels was noted in AgNPs-treated mice compared to control. No changes were observed among E 174-treated female mice.

In the liver, particles were only rarely seen in E 174-treated mice but present in AgNPs-treated mice. The presence of silver was only confirmed by TEM-EDX in particles in AgNPs-treated mice. There were no changes in inflammatory scores in limited H&E-stained tissue sections examined from E 174-treated mice but an increase in score in AgNPs-treated mice.

In the spleen, particles were only rarely seen in both E 174 and AgNPs-treated mice. However, silver was not detected by TEM-EDX in any particles examined. There were no changes in inflammatory scores in limited H&E-stained tissue sections examined from E 174-treated mice but evidence of injury and pathological changes in AgNPs-treated mice.

The author of the study stated that 'the study design was based on the following guidelines: sub-chronic oral toxicity testing based on OECD Guideline No. 408, Repeated-Dose 90-Day Oral Toxicity Study in Rodents (OECD, 2018); EFSA Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health'.[18] The Panel noted several major limitations, with the most notable being: (i) data on organs weight were not submitted; (ii) preservation of tissue for histology conducted only in few tissues (small intestine, Peyer's patches, colon, liver, spleen); (iii) haematological parameters and clinical biochemistry were not performed; (iv) microscopic examination was only carried out in intestine (jejunum and colon), liver and spleen; (v) histomorphological changes were only evaluated in the liver and spleen, and this evaluation was made only in 3 out of 10 animals per group per sex in the AgNPs group and in the highest dose E 174 group; (vi) hormone levels were not determined; (vii) parameters indicative

[18]The IBO referred to the EFSA Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health (EFSA Scientific Committee, 2018) available at the time of the testing, updated on 30 June 2021 (see references EFSA Scientific Committee, 2021b).

for endocrine effects were not evaluated. The Panel also noted that the author declared that the study was not performed following GLP principles.

Overall, the Panel considered this study limited in its ability to assess inflammatory changes in the limited number of organs examined. Taking into account that expression levels of only a limited selection of inflammatory-relevant cytokines were examined in the colonic mucosa of all animals, the Panel considered the changes to be either incidental or – in any case – adaptive and not necessarily indicative of an adverse effect.

In addition, the Panel noted that the relevance of the results for assessing the potential toxicity of nano-sized particles/aggregates/agglomerates cannot be verified (i.e. the relevance cannot be confirmed nor could it be excluded) because of the sample preparation; lack of the information on the degree of agglomeration of the pristine food additive and lack of information on the degree of agglomeration when incorporated into the feed. This situation creates uncertainty regarding the capacity of the study results to investigate the potential toxicity of the nano-sized particles/aggregates/agglomerates.

3.5 | Release of silver ions

The following was requested in the EC call for data[19]:

- Data on the release of silver ions from elemental silver in E 174: silver in food additive E 174 is present in its elemental form. There is evidence of the release of silver ions from elemental silver, which may be of concern. However, the extent of the release of silver ions, which depends on multiple factors such as pH and particle size, is unknown in the case of silver (E 174) used as food additive. Therefore, data on the release of silver ions from the additive (with fully characterised particle size), in aqueous media buffer in a range of pHs (1.5–7.4) at 37°C and at relevant concentrations and times, are requested.

No data were provided by the IBO on the release of silver ions from the food additive E 174 (with fully characterised particle size) as requested in the call for data.

4 | DISCUSSION

Silver (E 174) is a food colour that was re-evaluated by the ANS Panel in 2016 (EFSA ANS Panel, 2016). As a follow-up to that re-evaluation opinion, the EC issued a public call to gather data from IBOs that could reduce the uncertainties and the gaps in the dataset previously identified by the ANS Panel.

In response to the EC call for data, one IBO provided information on characterisation, manufacturing processes, particle size and morphology of silver used as a food additive (E 174).

Based on the data submitted, silver (E 174) contains not less than 99.5% (w/w) silver and up to 0.5% (w/w) of gold and/or copper. E 174 is described as 'a metallic material in the shape of small sheets and powders'. According to the information provided to EFSA, E 174 is produced by two different processes: (i) 'atomisation process' to produce silver powder and (ii) 'leaves process' to produce either silver leaves or powder (see Section 3.2.1). The IBO stated that E 174 resulting from both processes consists of 'homogenous, non-porous particles' having the shape of platelets with two relatively large and irregular dimensions and with one small and regular dimension representing the thickness.

The IBO provided limited information on particle size of E 174. Only one qualitative SEM image was provided for E 174 produced by the 'atomisation process' and no data were provided for E 174 produced as leaves by the 'leaves process'.

Incomplete quantitative information on particle size was provided for two samples of E 174 produced as powder by the 'leaves process'. The average thickness and the median thickness of 100 silver platelets were reported to be the same: 170 nm for one sample and 182 nm for the other sample. The fraction of particles (by number) with a thickness less than 100 nm was 0% and 3% for both samples.

The IBO proposed the following characterisation for E 174 in the EU specifications: a maximum of 10% of particles (by number) with one dimension below 100 nm and of 1% of particles (by number) with one dimension below 50 nm.

The IBO further stated that the variation of the smallest dimension of the particles is related to the variability of the manufacturing process and to the different production parameters of the silver leaves produced by the manufacturers. The Panel noted that the limited number of examined samples produced as powder by one manufacturing process might not be representative of all silver used as E 174 available on the EU market. The Panel further noted that the thresholds and maximum contents of particles by number proposed by the IBO for the E 174 specifications are not substantiated by analytical data. Furthermore, the Panel identified significant uncertainties in the data provided by the IBO, namely:

- The IBO did not provide quantitative information on the lateral dimensions of the silver platelets produced by the 'leaves process'.
- The IBO estimated that in E 174 the maximum level of particles with one dimension below 100 nm is 10%. However, information from the literature (De Vos et al., 2020; Waegeneers et al., 2019) showed that nanoparticles are released

[19] https://food.ec.europa.eu/document/download/fb9f972e-4767-4e7e-91c3-9e7fc9ff62d1_en?filename=fs_food-improvement-agents_reeval_call_20180410_e174_data.pdf.

from confectionary products containing E 174 when these were introduced into water. These studies found that 97% (by number) of the particles had at least one dimension smaller than 100 nm and a median size (D50) of 11 nm (TEM). The released silver particles were mostly of spheroidal morphology, even though the largest mass of silver was present as flakes with very rough edges (De Vos et al., 2020). Therefore, based on the results of these studies, the Panel considered that the percentage by number of nanoparticles released from E 174 in confectionary products is higher than what would be expected based on the results reported for the pristine material as investigated by the IBO.

- For the silver powder obtained by the 'atomisation process', the IBO did not provide any quantitative data on particle size distribution and only one SEM image was made available of which the resolution did not allow visualisation of constituent particles in the nano range. Upon request, no further clarifications on that aspect were provided by the IBO and therefore the Panel cannot exclude that the E 174 obtained in this process contains a much higher percentage than 10% of the particles with one dimension below 100 nm, as proposed in the specifications by the IBO.
- The submitted data do not provide information on the minimal external dimension (lowest measured thickness) of the silver platelets produced by both processes.
- It is not clear whether silver used as a food additive E 174 is coated or functionalised.

Clarification on all these aspects was not provided by the IBO in response to EFSA's request for additional information.

Based on the data presented above, the Panel is of the view that the data provided by the IBO on the characterisation of silver (E 174) were insufficient to fully characterise the materials used as a food additive. Consequently, the Panel is unable to propose specifications for silver (E 174) in relation to the morphology and size of the particles, as requested in the Terms of Reference (see Section 1.1).

Nonetheless, the Panel noted that based on the data provided (median (D50) of 170.2–182.4 nm), more than 10% of the particles of silver used as food additive E 174 produced as powder in the 'leaves process' has at least one dimension that is smaller than 250 nm. Therefore, considering this feature and following the EFSA Guidance on Particle-TR (EFSA Scientific Committee, 2021), the Panel is of the view that silver (E 174) contains small particles including nanoparticles.

Furthermore, the Panel noted that silver (silver wire, massive[20]) is considered insoluble in water (max 0.03 μg/L) (ECHA, 2024) and silver solubility varies depending on the particle size and shape. Particulate silver assessed by the SCCS consisting of spherical particles with a median particle size of circa 120 nm solubilises in water-buffered solutions at 22.8, 1.13 and 0.15 mg/L at pH 5, 7 and 9, respectively and it is considered insoluble in water (SCCS, 2024).

Therefore, in line with the EFSA Guidance on Particles -TR (EFSA Scientific Committee, 2021a), considering the silver solubility and the presence of the small particles including nanoparticles in E 174, the Panel is of the view that silver used as a food additive E 174 requires a risk assessment at the nanoscale following the EFSA Guidance on Nano-Risk Assessment (EFSA Scientific Committee, 2021b), to complement the conventional risk assessment performed according to the applicable sectorial guidance (EFSA ANS Panel, 2012).

The Panel further noted that no data were provided by the IBO on the release of silver ions from the food additive (with fully characterised particle size), as requested in the call for data.

Therefore, due to lacking information and remaining uncertainties regarding key properties of silver (E 174) (e.g. particle size distribution, shape, composition, silver ion release), which are essential to determine if silver materials tested in toxicity studies published in the open literature are relevant for the E 174 assessment, the Panel considered that conducting a literature search to identify additional evidence from the open literature to evaluate the safety of E 174, was not feasible. As a result, no literature search was performed as requested in the Terms of Reference of the present mandate and the current assessment considered only the data submitted by one IBO in response to the EC call for data.[21]

In the re-evaluation of silver (E 174) in 2016, the ANS Panel evaluated studies performed with AgNPs without differentiation of size and shape of the different tested materials and concluded that the available studies at that time provided clear evidence of a genotoxic potential in various in vitro test systems (EFSA ANS Panel, 2016). The in vivo oral genotoxicity data provided less conclusive evidence, and did not allow a definitive assessment of the possible genotoxic hazard associated with oral exposure to AgNPs.

Toxicological information in line with the tiered approach described in the EFSA's current guidance for submission for food additive evaluations (EFSA ANS Panel, 2012) was requested in the call for data for silver used as a food additive E 174. The IBO provided only data on genotoxicity and sub-chronic toxicity.

Regarding genotoxicity, the IBO provided only one in vitro and one in vivo study using the Prediscreen assay. The Panel noted that the Prediscreen assay, both in vitro and in vivo, is not validated for regulatory risk assessment, does not belong to the tests recommended in the current EFSA guidance documents and it is not included in the OECD testing guidelines recommended for the assessment of potential genotoxicity (OECD, 2017).

In addition, the Panel considered that the two studies are not reliable, and the sample preparation was not adequate for the assessment of particles at the nanoscale. The Panel considered the data presented by the IBO are of low relevance for the assessment of genotoxicity of E 174. Therefore, the Panel considered that the genotoxicity studies on E 174 provided by the IBO in response to the EC call for data do not fulfil the requirements for genotoxicity testing specified in the EFSA

[20]Silver wire, massive as reported in https://echa.europa.eu/fr/registration-dossier/-/registered-dossier/16155/4/9 accessed on 13/02/2025.

[21]https://food.ec.europa.eu/document/download/fb9f972e-4767-4e7e-91c3-9e7fc9ff62d1_en?filename=fs_food-improvement-agents_reeval_call_20180410_e174_data.pdf.

ANS Panel guidance (EFSA ANS Panel, 2012). In view of the presence of small particles including nanoparticles in E 174 genotoxicity data should have been generated following EFSA Guidance (EFSA Scientific Committee, 2018; EFSA Scientific Committee, 2021b).

A dietary sub-chronic toxicity study with E 174 in mice was submitted by the IBO. There were no clinical changes, no significant differences in food intakes or in body weights in any group throughout the study. Silver particles were only observed in the Peyer's patches present in the jejunum, not in the colon. No consistent and coherent changes in inflammatory scores in either the jejunum or colon regions were observed compared to the control group in E 174-treated animals. However, qualitative particle analyses and inflammatory scores were limited to three animals of each sex in the control and high dose E 174 groups. All animals were examined for changes in faecal lipocalin-2 and changes in the expression of selected cytokines in the colonic mucosa. There were no changes in faecal lipocalin-2 in all groups. In females exposed to E 174, a decrease in both pro- and anti-inflammatory cytokines was observed in colonic mucosa. In males, there was an increase in IL-10 and TNF-α but no increase in IL-1β. The Panel considered the anti-inflammatory cytokine IL-10 could selectively inhibit IL-1β expression without affecting TNF-α production. Overall, the Panel considered this study limited in its ability to assess inflammatory changes due to the limited number of organs examined. Taking into account that a limited selection of inflammatory-relevant cytokines expression levels was examined in the colonic mucosa of all animals, the Panel considered the changes to be either incidental or in any case adaptive and not necessarily indicative of an adverse effect.

Due to the insufficient information on the incorporation of silver (E 174) in the feed and the degree of aggregation/agglomeration therein as compared to E 174 in commercial foods, the Panel considered that the relevance of the results of the sub-chronic toxicity study for assessing nano-sized particles/aggregates cannot be verified (i.e. the relevance cannot be confirmed, nor could it be excluded). Specifically, any absence of an effect may be due to the lack of exposure to nano-sized particles/aggregates. This situation creates uncertainty regarding the capacity of the study to investigate the potential toxicity of the nano-sized particles/aggregates/agglomerates present in food available in the market.

Therefore, the Panel is of the view that the data obtained from the sub-chronic toxicity study, are not sufficient to conclude on the safety of the food additive.

Furthermore, the Panel considered that silver (E 174) tested in both genotoxicity assays and in the sub-chronic toxicity study, was produced as powder by the 'leaves process', which might not be representative of all types of silver used as food additive E 174 available on the market.

Overall, the Panel considered that the data submitted in the response to the EC call for data were insufficient. They lacked an adequate physicochemical characterisation of all types of silver used as E 174 available on the EU market, precluding a complementary assessment using data from the open literature. No data on the release of silver ions were submitted. The Panel also considered that the available genotoxicity data are inadequate to evaluate the genotoxic hazard associated with the use of silver (E 174). Furthermore, the Panel considered that the available toxicity data is not sufficient to assess the safety of silver used as a food additive E 174 according to the sectorial EFSA Guidance (EFSA ANS Panel, 2012) and the complementary and applicable guidance for the risk assessment of nanomaterials to be applied in the food and feed chain (EFSA Scientific Committee, 2021b).

5 | CONCLUSION

The Panel concluded that the technical data submitted by the IBO on the physicochemical characterisation of all types of silver used as food additive E 174 on the EU market were not adequate therefore, the Panel was unable to propose changes to the current EU specifications for silver (E 174) in relation to the morphology and size of the particles.

Due to the lack of adequate physicochemical characterisation of silver (E 174), the absence of data on the release of silver ions from E 174 and insufficient data on toxicity and genotoxicity, the Panel could not conclude on the safety of the food additive silver (E 174).

6 | DOCUMENTATION AS PROVIDED TO EFSA

1. Submission of data in response to the European Commission call for technical data on the permitted food additive silver (E 174). Submitted by Or A DÉCOR (Consortium of Producers of Edible Gold and Silver of Europe) on February 8, 2023.
2. PrediTox SAS, 2020, in vitro PrediScreen genotoxicity assay (γH2AX/p-H3 assay) with E 174 and AgNPs (comparison), Submitted by Or A DÉCOR (Consortium of Producers of Edible Gold and Silver of Europe) on February 8, 2023.
3. PrediTox SAS, 2021, in vivo PrediScreen genotoxicity assay (γH2AX/p-H3 assay) with E 174 and AgNPs (comparison) (tissue from the study: INREA, UMR 1331, Toxalim, 2021), Submitted by Or A DÉCOR (Consortium of Producers of Edible Gold and Silver of Europe) on February 8, 2023.
4. INREA, UMR 1331 Toxalim, (Research Centre in Food Toxicology) 2021. 13-week toxicity study by the oral route (test compound incorporated into food pellets) in male and female mice. Submitted by Or A DÉCOR (Consortium of Producers of Edible Gold and Silver of Europe) on February 8, 2023.

ABBREVIATIONS

AFM	atomic force microscopy
Ag^+	silver ion
AgNPs	silver nanoparticles
ANS Panel	EFSA Panel on Food Additives and Nutrient Sources added to Food
BET	Brunauer, Emmett and Teller
BPC	Biocidal Products Committee
CAS	Chemical Abstract Service
CLH	Harmonised Classification and Labelling
ECHA	European Chemicals Agency
EDX	Energy-dispersing X-ray Spectroscopy
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
GI	gastro-intestinal
GLP	Good Laboratory Practices
H&E	Haematoxylin and Eosin
IBO	interested business operator
IL-10	interleukin-10
IL-1β	interleukin-1 beta
Lcn-2	lipocain-2
NM	nanomaterial
No	Number
NOAEL	no observed adverse effects level
OECD	Organisation for Economic Co-operation and Development
qPCR	quantitative Polymerase Chain Reaction
RAC	Risk Assessment Committee
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals Regulation
ROS	reactive oxygen species
SCCS	Scientific Committee on Consumer Safety
SD	standard deviation
SEM	scanning electron microscope
SP-ICP-MS	single particle inductively coupled plasma mass spectrometry
TEM	transmission electron microscope
TNF-α	tumour necrosis factor alpha
VSSA	volume specific surface area
w/w	Weight per weight

REQUESTOR

European Commission

QUESTION NUMBER

EFSA-Q-2023-00169

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PANEL MEMBERS

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

COMPETING INTERESTS

In line with EFSA's policy on declarations of interest, Panel member Eric Houdeau did not participate in the development and adoption of this scientific output.

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APPENDIX A

Summary tables of the genotoxicity studies

TABLE A.1 Reporting table on the assessment of relevance and reliability of genotoxicity studies on silver (E 174).

Test system	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/Comments	Relevance of test system/Relevance of the result	Reference
Other In vitro assays						
In vitro γH2AX/ p-H3 assay (PrediScreen Assay)[22] Human cell lines in vitro: HepG2 (liver), LS-174T (colon), ACHN (kidney)	Concentrations E 174: 20, 40, 80 and 160 µg/mL AgNPs: 80 and 160 µg/mL Solvent: ultrapure water – negative controls: DMSO, ultrapure water. – positive controls γH2AX: HepG2 and LS-174T: benzo[α] pyrene (1 µM), ACHN: etoposide (10 µM) p-H3: All cell lines: nocodazole (1 µM) Treatment time: 24 h Oxidative stress model compounds: Antioxidant: N-acetylcysteine (NAC); Pro-oxidant: Buthionine sulfoximine (BSO) Treatment time: 1, 2, 4, 24 h – Negative controls: DMSO, ultrapure water – Positive controls: benzo[α] pyrene (1 µM), menadione (10 µM) Triplicates; Student's *t*-test Cytotoxicity (relative cell count, %RCC)	E 174 (powder)[23] Silver nanoparticles (AgNPs) (SkySpring Nanomaterials, Inc. (Houston, USA))[23] Stock solutions: E 174: 2 mg/mL in ultrapure water. Sonicated (for 10 min) and non-sonicated. Stored at +4°C. AgNPs: 2 mg/mL in ultrapure water. Sonicated (for 10 min). Stored at +4°C.	p-H3 induction a. E 174 sonicated: no effect (all cell lines; all 4 doses) b. E 174 not-sonicated: no effect (all cell lines; all 4 concentration) c. AgNPs sonicated: no effect (all cell lines; all 2 doses) γH2AX induction a. E 174 sonicated: concentration-dependent increase at 80 and 160 µg/mL in HepG2 cells ($p < 0.01$). d. E 174 not-sonicated: no effect (all cell lines; all 4 concentration). b. AgNPs sonicated: increase at 160 µg/mL in HepG2 cells ($p < 0.01$). Oxidative stress (induction of γH2AX, HepG2; E 174 sonicated) a. E 174 sonicated: 1 h, 2 h: no effect. 4 h: increase at 160 µg/mL ($p < 0.01$). 24 h: concentration-dependent increase at 80 and 160 µg/mL ($p < 0.05$). b. E 174 sonicated+NAC: 1 h, 2 h: no effect. 4 h: increase at 160 µg/mL ($p < 0.01$). 24 h: concentration-dependent increase at 80 and 160 µg/mL ($p < 0.05$). c. E 174 sonicated+BSO: 1 h, 2 h: no effect. 4 h: concentration-dependent increase at 80 and 160 µg/mL ($p < 0.01$). 24 h: concentration-dependent increase at 80 and 160 µg/mL ($p < 0.01$). Cytotoxicity No cytotoxicity was observed. E 174 sonicated: increase in cell viability (due to particles attached to the wells; not considered a treatment effect).	Not reliable (score 3) – Test not validated for regulatory Risk Assessment. – Not GLP study – Sample preparation including sonication process is not described	Low/Low	Documentation provided to EFSA No. 2

(Continues)

[22]Sample is considered genotoxic if it induces a statistically significant increase in the biomarker > 30% and if the cytotoxicity is less than 50%. Sample not considered genotoxic if the induction value is 0.7–1.3.

[23]The Panel noted that E 174 and AgNPs used in the in vitro study were not characterised, however based on the description in the study the Panel considered that these materials are the same as used in the in vivo study (see table below).

TABLE A.1 (Continued)

Test system	Exposure conditions	Information on the characteristics of the test substance	Result	Reliability/Comments	Relevance of test system/Relevance of the result	Reference
Other In vivo assays						
in vivo γH2AX/ p-H3 assay (PrediScreen Assay) 13 weeks oral (dietary) study C57BL/6J mice 10 mice/sex/group Tissues: Colon (segment of intestinal tissue), liver (right median lobe)	Repeated oral (food admixture) toxicity study Doses E 174: 0, 1, 10, 100 µg/kg bw per day (human relevant doses according to EFSA ANS, 2016, see also Appendix B.) AgNPs: 100 µg/kg bw per day Exposure 90 days (13 weeks) Analysis 200,000 cells/mL per tissue were tested, in duplicate in 96-well plates (40,000 cells/well). γH2AX/p-H3 assay Student's t-test Controls: – Positive control not mentioned, – Negative control (no addition of tested substance in the food)	E 174 produced as powder by the leaves process. The sample characterisation was submitted (see details in Section 3.2.3, Table 2, sample 2). Silver nanoparticles (AgNPs, 50 nm diameter, SkySpring Nanomaterials, Inc. (Houston, USA)).	<u>Genotoxicity</u> Colon samples: p-H3 induction a. E 174: no effect (all three doses, males and females). b. AgNPs: no effect (in one dose tested, males and females). γH2AX induction a. E 174: Statistically significant increase of γH2AX at all doses (1*, 10**, 100** µg/kg bw per day) in females (dose-dependent effect) and at 10 and 100 µg/kg bw per day in males (no dose-dependent). b. AgNPs: statistically significant increase of γH2AX at 100** µg/kg bw per day in both males and females. (*$p < 0.05$; **$p < 0.01$) <u>Liver samples:</u> p-H3 induction a. E 174: no effect (all three doses, males and females). b. AgNPs: no effect (in one dose tested, males and females). γH2AX induction a. E 174: no effect (all three doses, males and females). b. AgNPs: no effect (in one dose tested, males and females).	– Not reliable (score 3) – Test not validated for regulatory Risk Assessment. – Not GLP study – Insufficient information on the incorporation of silver used as E 174 in the feed and the degree of aggregation/ agglomeration therein as compared to the commercial foods, – No positive controls used. – Post-treatment period not mentioned.	Low/Low	Documentation provided to EFSA No. 3

APPENDIX B

Summary of the sub-chronic oral toxicity study in mice

The sub-chronic study was performed using: (i) E 174 produced as powder by the 'leaves process' for which the information concerning particle size and morphology were provided as reported in Section 3.2.3 (see Table 2, sample 2), as well as on (ii) silver nanoparticles (AgNPs, spherical particles, size 50–60 nm). Five-week-old male and female C57BL/6J mice (10/sex/group) were given silver (E 174) in the diet (at concentrations corresponding to doses of 0, 1, 10 or 100 μg/kg bw per day) or AgNPs (0 or 100 μg/kg bw per day) for 90 days (Documentation provided to EFSA N 4). The study authors justified the selection of the doses to be tested based on the human dietary exposure to E 174 estimated at the time of the re-evaluation (EFSA ANS Panel, 2016). The test items, E 174 and AgNPs were incorporated to the feed pellet by admixture and pellet formulation by compaction. No further information was provided on the preparation of the feed with E 174 and AgNPs. No information on the degree of aggregation/agglomeration of silver particles in the feed and no comparison to the aggregation/agglomeration of E 174 as present in marketed food was available. The Panel noted that also no information on the homogeneity of both E 174 and AgNPs in the feed was provided.

The presence of particles in tissues (jejunum, Peyer's patches, colon, liver, spleen) was investigated using confocal laser diffraction (detecting inorganic laser-reflecting particles, not necessarily Ag) and TEM-EDX (that can distinguish Ag-containing particles). The Panel noted that some difficulties may be encountered when TEM-EDX detection is used to chemically identify particles smaller than 20 nm due to the low spatial resolution of this characterisation technique.

Test items were well tolerated, no clinical signs were observed throughout the study and no morphological (macroscopic) abnormalities were observed on the day of sacrifice in the abdominal and pelvic organs. There were no deaths throughout the study, no significant differences in food intake and in body weight.

In the study, effects on the composition of the gut microbiota (based on stool analysis) by the tested items were investigated and some changes in gut microbiota metabolic activity observed. E 174 exposure had limited effects on the composition of the gut microbiota in male mice, but it did cause a metabolic imbalance with increased medium-chain fatty acids (MCFAs) levels at 10 and 100 μg/kg bw per day (+163.6% and +310.2%, respectively compared to controls) and decreased acetate levels (−42.8%) at 100 μg/kg bw per day. In AgNPs-treated males, a significant (56%) decrease in the production of MCFAs occurred.

E 174 altered significantly the gut microbiota composition in female mice at 1 and 100 μg/kg bw per day.

Similar to males, AgNPs nanomaterial did not significantly affect gut microbiota composition in females.

GI-jejunum

No laser-reflecting metal particles were observed in the jejunum of control and high dose E 174-treated mice. AgNPs-treated mice showed a few laser-reflecting particles in the villi, reaching the lamina propria. Particles were absent or rarely seen in the Peyer's patches in all groups exposed to E 174. No quantitative data was included in the report.

TEM-EDX spectroscopy identified the presence of silver in Payer's patch M cells in E 174 mice (where particles in the microvilli of enterocytes were examined). Silver was detected in the cytoplasm of enterocytes near the basolateral membrane and in the cytoplasm of Payer's patch M cells in AgNPs-treated mice. Analysis with control tissues was not reported.

Ileal barrier permeability (to Fluorescein isothiocyanate (FITC) conjugated 4 kDa dextran) and trans-epithelial resistance were tested on all males and females in all treatment groups.

An apparent (non-statistically significant) increase in the lumen-to-mucosal flux of FITC- dextran was noted only in the ileum of males treated with 1 μg/kg bw per day E 174 relative to the control group in the absence of any change in trans-epithelial resistance. In the absence of any change in trans-epithelial resistance in this group, the Panel considered this apparent change in lumen-to-mucosal flux of FITC- dextran incidental. No changes in permeability were seen in females in any treatment group.

No changes in lumen-to-mucosal flux of FITC- dextran or in trans-epithelial resistance was seen in AgNPs-treated animals.

Inflammatory scores (based on microscopic examination of H&E-stained tissue sections from the control and high-dose groups only; only three animals/sex/examined) indicated that there was no significant difference in the jejunal mucosa between control animals and high dose E 174 or AgNPs groups.

GI-colon

Laser-reflecting particles were absent or rarely seen in the colon in control and high dose E 174-treated animals. In the AgNPs-treated animals, laser-reflecting particles were observed in the mucosa. No quantitative data was included in the report.

TEM-EDX spectroscopy of electron-dense particles identified particles in the lamina propria as well as the cytoplasm of enterocytes of E 174-treated mice and numerous inorganic particles in epithelial cells (enterocytes and mucus-producing goblet cells) in AgNPs-treated animals. Silver was not detected in all cases. Analysis with control tissues was not reported.

Colonic barrier permeability (to FITC-conjugated 4 kDa dextran) and trans-epithelial resistance were tested on all males and females in all treatment groups. No changes in lumen-to-mucosal flux of FITC- dextran or in trans-epithelial resistance were seen in any treatment groups.

Inflammatory scores (based on microscopic examination of H&E-stained tissue sections from the control and high dose groups only; only three animals/sex/examined) indicated that 1 of the 6 E 174-treated mice showed weak inflammation with tissue lesions but the inflammatory score for this group was not significantly different from the control group. In contrast, a statistically significant increased inflammatory score was reported for the AgNPs-treated animals (moderate inflammation was seen in four of the six AgNPs-treated animals examined). Some particles were observed in the mucus cells of this group.

To characterise the inflammatory response in the intestine, the expression and production of pro- and anti-inflammatory cytokines by the colonic mucosa were evaluated using qPCR (RNA assay) and ELISA (protein assay), respectively. The evaluation was conducted on all animals. As pro-inflammatory cytokines: interleukin (IL)-1β, IL-17, tumour necrosis factor (TNF-α) and interferon (IFN)-γ were measured. As anti-inflammatory markers: IL-10 and transforming growth factor (TGF)-β were measured. In addition, levels of lipocalin-2 (Lcn-2) were measured in faeces of all animals to assess the overall inflammatory response in the intestine.

Concerning pro-inflammatory cytokines, in males a decrease in IL-1β protein was observed in animals exposed to AgNPs and E 174 (1 and 10 μg/kg bw per day dose groups), which was paralleled by a decrease in mRNA relative expression. A statistically significant increase in TNF-α protein was observed at all doses, paralleled by an increase in TNF-α mRNA relative expression. While the effects at the protein level were not dose-related, the effects at the mRNA were. No changes in IFN-γ or IL-17 were registered in both AgNPs and E 174-treated mice. Concerning anti-inflammatory cytokines, no changes in TGF-β were reported, while a statistically significant decrease in IL-10 protein was observed in AgNPs-treated mice and an increase at E 174 in the 100 μg/kg bw per day dose group. The effect at the protein level was not paralleled by a change in mRNA expression.

In females, AgNPs and E 174 induced a statistically significant decrease in both IL-1β and TNF-α protein at all doses, partially paralleled by a decrease at the mRNA levels. No changes in IFN-γ or IL-17 were registered in both AgNPs and E 174-treated mice. Concerning anti-inflammatory cytokines, no changes in TGF-β were reported, while a statistically significant decrease in IL-10 protein was observed in all groups. The effect at the protein level was paralleled by a change in mRNA expression.

Decreased IL-1β and increased TNF-α observed in males could reflect a specific immune response shift or a pathological state depending on the biological context. These cytokines, although both pro-inflammatory, have distinct roles in immune regulation and inflammation. IL-1β downregulation suggests a reduction in inflammasome activation or early-stage inflammatory signalling. While increased TNF-α may indicate a sustained or chronic inflammatory state where TNF-α dominates the inflammatory response, possibly driven by macrophages or T cells. Thus, the immune system may be in a state where TNF-α is driving tissue damage or chronic inflammation while IL-1β is being regulated to prevent excessive early inflammatory responses. The increase in TNF-α in males may parallel the colon inflammatory scores observed microscopically, showing slight effects in males (1.33 ± 0.58 vs. 0.33 ± 0.58 in control mice, $n = 3$). Gender differences in the modulation of intestinal IL-1β and TNF-α production are likely due to hormonal, genetic and microbial factors.

Overall, in females exposed to E 174, decrease in both pro- and anti-inflammatory cytokines were observed. While in males, the anti-inflammatory cytokine IL-10 could selectively inhibiting IL-1β without affecting TNF-α production.

Regarding Lcn-2 levels, no changes were observed in both AgNPs- and E 174-treated male mice. In females, a slight but not statistically significant increase in faecal Lcn-2 levels was noted in mice exposed to the AgNPs compared to control. No changes were observed among E 174-treated female mice. Despite the colonic tissue damage observed microscopically in the AgNPs-treated mice compared to controls (inflammatory score 2.33 ± 0.49 vs. 0.33 ± 0.21 in control mice, $p < 0.01$, $n = 6$, pooled three animals per sex), the absence of significant effects on Lcn-2 levels raises questions about its sensitivity as a biomarker in this context. The absence of effects on Lcn-2 levels aligned more closely with the lack of changes in intestinal permeability or disruption of epithelial integrity in male and female mice at the end of the treatment period.

Liver

No laser-reflecting particles were observed in the liver of control animals. In high dose E 174-treated animals, particles were rarely observed. In the AgNPs-treated animals, laser-reflecting particles were observed close to granulomas. No quantitative data was included in the report.

TEM-EDX spectroscopy of electron-dense particles in the cytoplasm of hepatocytes from E 174-treated animals did not identify silver. AgNPs-treated mice particles present in cytoplasm and close to the nuclei of hepatocytes did contain silver. Analysis with control tissues was not reported.

Inflammatory scores and 'histomorphological changes' (based on microscopic examination of H&E-stained tissue sections from the control and high dose groups only; only three animals/sex/examined) were also assessed in the liver.

In 3 out of the 6 E 174-treated mice, granulomas were observed. However, granulomas were also found in two of the six control mice and it was concluded by the author that the granulomas in the E 174 group were not treatment-related.

'Aberrant cell morphological features and/or foci of inflammatory cells' were seen in the liver of six out of six AgNPs-treated animals. No statistical analyses were performed on these data.

Spleen

No laser-reflecting particles were observed in the spleen of control animals. In high dose E 174-treated animals, a few particles were observed in the red pulp. In the AgNPs-treated animals, laser-reflecting particles were rarely observed. No quantitative data was included in the report.

TEM-EDX spectroscopy of electron-dense particles did not identify silver in both E 174- and AgNPs-treated animals. Analysis with control tissues was not reported.

In the spleen (based on microscopic examination of H&E-stained tissue sections from the control and high dose groups only; only three animals/sex/examined) there was no difference reported between E 174-treated animals and control animals (1/6 vs. 2/6 demonstrating 'injury' respectively). In AgNPs-treated animals, 'brownish pigments' were observed in macrophages and 4/6 animals were reported as showing injury. No statistical analyses were performed on these data.