Physico-chemical characterisation of the fraction of silver (nano)particles in pristine food additive E174 and in E174-containing confectionery

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\section*{ABSTRACT}
Silver (E174) is authorised as a food additive in the EU. The unknown particle size distribution of E174 is a specific concern for the E174 risk assessment. This study characterised the fraction of silver (nano) particles in 10 commercially available pristine E174 food additives and 10 E174-containing products by transmission electron microscopy (TEM) and single-particle inductively coupled plasma-mass spectrometry (spICP-MS). TEM analysis showed that all samples contained micrometre-sized flakes and also a fraction of (nano)particles. Energy-dispersive X-ray spectroscopy (EDX) and electron diffraction confirmed that the (nano)particles and micrometre-sized flakes consisted of silver. A higher amount of (nano)particles was observed in the products than in the food additives. In addition, the surface of the micrometre-sized flakes was rougher in products. The median of the minimum external dimension, assessed as minimal Feret diameter, of the fraction of (nano)particles determined by quantitative TEM analysis was 11 ± 4 nm and 18 ± 7 nm (overall mean ± standard deviation), for food additives and products, respectively. Similar size distributions were obtained by spICP-MS and TEM, considering the limit of detection of spICP-MS. The median of the equivalent spherical diameter of the fraction of (nano)particles determined by spICP-MS was 19 ± 4 nm and 21 ± 2 nm (overall mean ± standard deviation), for food additives and products, respectively. In all samples, independent of the choice of technique, the nano-sized particles represented more than 97\% (by number) of the silver particles, even though the largest mass of silver was present as flakes.

\section*{Introduction}
Food additive E174 (silver) is permitted for use in food in the European Union (EU) at quantum satis, for the external coating of confectionery, for the decoration of chocolates and in liqueurs (EC 2008). Silver in food additive E174 is assumed to be present in its elemental form (EC 2012), and Verleysen et al. (2015) and de la Calle et al. (2018) have demonstrated the presence of silver nanoparticles (Ag NPs) in E174-containing confectionery. Other studies aiming to characterise Ag NPs in food mainly treated samples mimicking ‘real’ food products, such as spiked chicken meat containing a fraction of Ag NPs (Loeschner et al. 2013; Peters et al. 2014; Dudkiewicz et al. 2015).

Studies on non-food silver have demonstrated that toxicity depends on the reactivity of the silver form and on its potential to release silver ions through oxidation (Liu et al. 2010; Levard et al. 2013). The size of the silver nanoparticles demonstrated by Verleysen et al. (2015) in E174-containing products allows endocytic uptake by the cells of the human intestine (Miethling-Graff et al. 2014; Georgantzopoulou et al. 2015; Vila et al. 2018). Silver nanoparticles are known to be unstable and subject to transformation depending on their surroundings (Ahlberg et al. 2014; Potter et al. 2019). Transformations of Ag NPs were shown to occur as they move through the gastrointestinal (GI) tract (Walczak et al. 2012; Marchioni et al. 2018), which influences the uptake of silver in GI tissues. These reported mechanisms shown for colloidal silver particles deserve in-depth examination for silver particles in E174 as well. Studies showing the relation between Ag NP size and dissolution rate (Liu et al. 2010; Ma et al. 2011) stress the importance of knowledge of the particle size distribution, since the hazard associated with nano-silver depends on the size of the particles.
In the opinion of the European Food Safety Authority (EFSA) on the re-evaluation of E174 used as a food additive, the Panel concluded that the available information was insufficient to assess the safety of silver as food additive (EFSA 2016). The major issues included chemical identification and characterisation of E174, including unknown particle size distributions, the unknown quantity of nanoparticles and release of ionic silver, and the lack of similar information on the material used in the available toxicity studies. In addition, EFSA’s guidance document for nanotechnology and nanoscience in the food and feed chain states that other relevant characteristics of the nano-scale fraction should be examined, such as complex transformations and de novo formation of particles from ionic species, which affect the toxicity of food (EFSA 2018). Food matrix components may alter the physico-chemical characteristics of Ag NPs present in food (Martirosyan et al. 2016).

To address, at least partly, the knowledge gaps discussed in the EFSA opinion on the re-evaluation of E174 ([EFSA] European Food Safety Authority 2016), this work presents the results of the physico-chemical characterisation of the fraction of (nano) particles in 10 pristine E174 food additives (hereafter referred to as food additives), and 10 confectionery products containing E174 (hereafter referred to as products), purchased from several European web shops and Belgian supermarkets. This study is more elaborate than the limited case studies of Verleysen et al. (2015) and de la Calle et al. (2018), and allows comparing the particle size distribution, shape, surface properties and structure of E174 in food additives and products. In view of the reactivity of silver and its capability to release silver ions and form de novo Ag NPs, sample preparation artefacts caused by the dispersion medium and by probe sonication are evaluated. According to EFSA’s guidance document for nanotechnology and nanoscience in the food and feed chain ([EFSA] European Food Safety Authority 2018) and the European Committee for Standardisation (CEN) guidelines (CEN 2018), the particle size distributions are determined by two independent techniques: quantitative transmission electron microscopy (TEM) and single-particle inductively coupled plasma-mass spectrometry (spICP-MS). Stability is monitored based on the crystal structure and elemental composition measured by electron diffraction and energy-dispersive X-ray spectroscopy (EDX), respectively. Inductively coupled plasma-optical emission spectroscopy (ICP-OES) and spICP-MS are applied to determine the total silver concentration and the concentration of Ag NPs, respectively.

Materials and methods

Materials

A powdered silver material, manufactured by Laboratorios Argenol S.L. (Zaragoza, Spain) and distributed by ABC Chemicals (Nazareth, Belgium) was used as a positive control for method optimisation and is referred to as Ag-001 (Table 1). Although it is difficult to classify E174 based on its applications, for pragmatic reasons a distinction is made between the pristine food additives and products, based on the absence or presence of other ingredients. A direct link between the starting material and the products as described by Geiss et al. (2020) for E171 could not be established in this study. Ten food additives, referred to as Ag-002 to Ag-011, were purchased online from producers and suppliers from several European countries. These included powders (≤1 mm), flakes and petals (1 mm–2 cm), and leaves (>2 cm) (Table 1). Ag-002 is a mixture of food additives. It was classified and treated as a food additive since it did not contain other food matrix components such as sugar or chocolate. Ten products, referred to as Ag-P-001 to Ag-P-010, were purchased in Belgian food stores. These products included a variety of confectionery such as silver-coated sugar beans and silver pearls (Table 1). A complete list of ingredients, as cited on the label, is shown in the supplementary information (S1).

Sample preparation

In the sample preparation protocols applied to the food additives and products, probe sonication was applied using a Vibra-cell”75041 ultrasonifier (750 W, 20 kHz, Fisher Bio-block Scientific, Aalst, Belgium) equipped with a 13 mm horn (CV33) at 40% amplitude. The sonicator was calibrated as described in the standard operating protocol (SOP) for ‘probe-sonicator calibration of delivered
<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Components</th>
<th>Ferret min(^1) (nm)</th>
<th>Ferret min(^2) (nm)</th>
<th>ESD (nm)</th>
<th>Aspect ratio</th>
<th>Solidity</th>
<th>(C_m) (g kg(^{-1}))</th>
<th>(C_p) (kg (^{-1}))</th>
<th>Total mass (g/kg)</th>
<th>mass % NP(^9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag-001 Powder</td>
<td>Silver</td>
<td>E174</td>
<td>13 ± 2</td>
<td>13 ± 2</td>
<td>14 ± 3</td>
<td>1.07 ± 0.16</td>
<td>0.98 ± 0.15</td>
<td>385 ± 129</td>
<td>2.0 ± 0.7 × 10(^{10})</td>
<td>NA(^6)</td>
<td>38 ± 14</td>
</tr>
<tr>
<td>Ag-002 Powder (0.2 mm)</td>
<td>E174, E202, E414</td>
<td>12 ± 2</td>
<td>NA(^6)</td>
<td>ND(^3)</td>
<td>1.14 ± 0.17</td>
<td>0.97 ± 0.15</td>
<td>ND(^3)</td>
<td>NA(^6)</td>
<td>ND(^3)</td>
<td>NA(^6)</td>
<td>ND(^3)</td>
</tr>
<tr>
<td>Ag-003 Powder (1 mm)</td>
<td>E174</td>
<td>12 ± 2</td>
<td>NA(^6)</td>
<td>16 ± 5</td>
<td>1.29 ± 0.20</td>
<td>0.97 ± 0.15</td>
<td>2.2 ± 0.8</td>
<td>3.0 ± 1.2 × 10(^{10})</td>
<td>0.24 ± 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-004 flakes (2 mm)</td>
<td>E174</td>
<td>14 ± 3</td>
<td>ND(^3)</td>
<td>10 ± 2</td>
<td>1.27 ± 0.09</td>
<td>0.97 ± 0.15</td>
<td>1.9 ± 0.6</td>
<td>1.3 ± 0.4 × 10(^{10})</td>
<td>0.19 ± 0.08</td>
<td></td>
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</tr>
<tr>
<td>Ag-005 flakes (2 mm)</td>
<td>E174</td>
<td>14 ± 3</td>
<td>ND(^3)</td>
<td>10 ± 2</td>
<td>1.27 ± 0.09</td>
<td>0.97 ± 0.15</td>
<td>1.9 ± 0.6</td>
<td>1.3 ± 0.4 × 10(^{10})</td>
<td>0.19 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-006 petals (1 cm)</td>
<td>E174</td>
<td>14 ± 3</td>
<td>ND(^3)</td>
<td>10 ± 2</td>
<td>1.27 ± 0.09</td>
<td>0.97 ± 0.15</td>
<td>1.9 ± 0.6</td>
<td>1.3 ± 0.4 × 10(^{10})</td>
<td>0.19 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-007 petals (1.5 cm)</td>
<td>E174</td>
<td>14 ± 3</td>
<td>ND(^3)</td>
<td>10 ± 2</td>
<td>1.27 ± 0.09</td>
<td>0.97 ± 0.15</td>
<td>1.9 ± 0.6</td>
<td>1.3 ± 0.4 × 10(^{10})</td>
<td>0.19 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-008 leaves (8 cm)</td>
<td>E174</td>
<td>14 ± 3</td>
<td>ND(^3)</td>
<td>10 ± 2</td>
<td>1.27 ± 0.09</td>
<td>0.97 ± 0.15</td>
<td>1.9 ± 0.6</td>
<td>1.3 ± 0.4 × 10(^{10})</td>
<td>0.19 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-009 leaves (9.5 cm)</td>
<td>E174</td>
<td>14 ± 3</td>
<td>ND(^3)</td>
<td>10 ± 2</td>
<td>1.27 ± 0.09</td>
<td>0.97 ± 0.15</td>
<td>1.9 ± 0.6</td>
<td>1.3 ± 0.4 × 10(^{10})</td>
<td>0.19 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-010 flakes</td>
<td>E174</td>
<td>14 ± 3</td>
<td>ND(^3)</td>
<td>10 ± 2</td>
<td>1.27 ± 0.09</td>
<td>0.97 ± 0.15</td>
<td>1.9 ± 0.6</td>
<td>1.3 ± 0.4 × 10(^{10})</td>
<td>0.19 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-011 flakes</td>
<td>E174</td>
<td>14 ± 3</td>
<td>ND(^3)</td>
<td>10 ± 2</td>
<td>1.27 ± 0.09</td>
<td>0.97 ± 0.15</td>
<td>1.9 ± 0.6</td>
<td>1.3 ± 0.4 × 10(^{10})</td>
<td>0.19 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-P-001 sugar beans</td>
<td>E100, E122, E124, E171, E174</td>
<td>17 ± 3</td>
<td>NA(^6)</td>
<td>24 ± 4</td>
<td>1.22 ± 0.19</td>
<td>0.98 ± 0.15</td>
<td>0.21 ± 0.007</td>
<td>7.1 ± 2.4 × 10(^{10})</td>
<td>0.39 ± 0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-P-002 sugar pearls</td>
<td>E100, E122, E124, E171, E174</td>
<td>17 ± 3</td>
<td>NA(^6)</td>
<td>24 ± 4</td>
<td>1.22 ± 0.19</td>
<td>0.98 ± 0.15</td>
<td>0.21 ± 0.007</td>
<td>7.1 ± 2.4 × 10(^{10})</td>
<td>0.39 ± 0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-P-003 sugar beans</td>
<td>E100, E122, E124, E171, E174</td>
<td>17 ± 3</td>
<td>NA(^6)</td>
<td>24 ± 4</td>
<td>1.22 ± 0.19</td>
<td>0.98 ± 0.15</td>
<td>0.21 ± 0.007</td>
<td>7.1 ± 2.4 × 10(^{10})</td>
<td>0.39 ± 0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-P-004 sugar beans</td>
<td>E100, E122, E124, E171, E174</td>
<td>17 ± 3</td>
<td>NA(^6)</td>
<td>24 ± 4</td>
<td>1.22 ± 0.19</td>
<td>0.98 ± 0.15</td>
<td>0.21 ± 0.007</td>
<td>7.1 ± 2.4 × 10(^{10})</td>
<td>0.39 ± 0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-P-005 sugar beans</td>
<td>E100, E122, E124, E171, E174</td>
<td>17 ± 3</td>
<td>NA(^6)</td>
<td>24 ± 4</td>
<td>1.22 ± 0.19</td>
<td>0.98 ± 0.15</td>
<td>0.21 ± 0.007</td>
<td>7.1 ± 2.4 × 10(^{10})</td>
<td>0.39 ± 0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-P-006 sugar beans</td>
<td>E100, E122, E124, E171, E174</td>
<td>17 ± 3</td>
<td>NA(^6)</td>
<td>24 ± 4</td>
<td>1.22 ± 0.19</td>
<td>0.98 ± 0.15</td>
<td>0.21 ± 0.007</td>
<td>7.1 ± 2.4 × 10(^{10})</td>
<td>0.39 ± 0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-P-007 sugar beans</td>
<td>E100, E122, E124, E171, E174</td>
<td>17 ± 3</td>
<td>NA(^6)</td>
<td>24 ± 4</td>
<td>1.22 ± 0.19</td>
<td>0.98 ± 0.15</td>
<td>0.21 ± 0.007</td>
<td>7.1 ± 2.4 × 10(^{10})</td>
<td>0.39 ± 0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-P-008 sugar pearls</td>
<td>E174</td>
<td>23 ± 4</td>
<td>NA(^6)</td>
<td>21 ± 3</td>
<td>1.28 ± 0.20</td>
<td>0.98 ± 0.15</td>
<td>0.0020 ± 0.00010</td>
<td>1.7 ± 0.6 × 10(^{10})</td>
<td>0.41 ± 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-P-009 sugar pearls</td>
<td>E174</td>
<td>23 ± 4</td>
<td>NA(^6)</td>
<td>21 ± 3</td>
<td>1.28 ± 0.20</td>
<td>0.98 ± 0.15</td>
<td>0.0020 ± 0.00010</td>
<td>1.7 ± 0.6 × 10(^{10})</td>
<td>0.41 ± 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag-P-010 sugar beans</td>
<td>E174, E100</td>
<td>10 ± 2</td>
<td>NA(^6)</td>
<td>22 ± 4</td>
<td>1.12 ± 0.17</td>
<td>0.98 ± 0.15</td>
<td>0.0060 ± 0.0020</td>
<td>2.0 ± 0.7 × 10(^{10})</td>
<td>0.35 ± 0.14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)The list of main food additives in products (Ag-001-Ag-007) is shown depending on the colour of products provided by the manufacturer. For some products (Ag-008-Ag-009) the list of components is given as such. Food products have an interior consisting of major components chocolate (or cacao) and sugar. The outer coating of the food products consisted of Ag-174. A coating of TiO\(_2\) (E171) enclosing the inner core was found beneath the Ag coating for some products. A complete list of components is given in Table S1.

\(^2\)EM results of non-sonicated E174 dispersions

\(^3\)Not detectable, because no sufficient number of particles could be measured

\(^4\)Calculated for routine TEM analyses performed on 2 days

\(^5\)TEM results of sonicated E174 dispersions

\(^6\)Not applicable

\(^7\)Nanoparticle mass concentration of NPs with a size below 100 nm as determined by ICP-MS. \(C_m\) is expressed in mg meter\(^{-3}\) or whole product, in food additives and products, respectively.

\(^8\)Nanoparticle number concentration of NPs with a size below 100 nm as determined by ICP-MS expressed per kilogram additive or whole product

\(^9\)Mass percentage of NPs with a size below 100 nm per total mass of silver determined by ICP-OES (products) or assumed to be equal to 1000 g/kg (food additives). Measurement results are presented with their expanded measurement uncertainty (U, k = 2) under routine measurement conditions.
acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing of the NANOGENOTOX project (NANOGENOTOX 2016). All samples were cooled in water with ice to prevent excessive heating during sonication.

Prior to TEM analysis, material Ag-001 was dispersed in double-distilled water at a concentration of 2.56 mg/mL and sonicated until 7 ± 2 kJ of energy was added. The food additives, Ag-002 to Ag-011, were prepared in duplicate, following the generic NANOGENOTOX dispersion protocol (Jensen et al. 2011). Approximately 15 mg of each food additive was brought into a 20 mL liquid scintillation vial (Wheaton, Millville, NJ, USA), wetted drop-wise with 30 μL ethanol (96%) and 0.970 mL of a 0.05% w/v sterile-filtered bovine serum albumin (BSA) solution (cooled overnight), after which 5 mL of 0.05% w/v sterile-filtered BSA solution was added to reach a final concentration of 2.56 mg/mL. One replicate was sonicated for 16 min until an energy of 37 ± 1 kJ was added. For the second replicate, probe sonication was omitted. The products, Ag-P-001 to Ag-P-010, have a core mainly consisting of sugar or chocolate, covered by a silver coating. To avoid effects of sample preparation, dispersions of products were prepared by isolating the entire silver coating of a random amount of pearls by elution in water as applied in Verleysen et al. (2015). No sonication was applied. The samples were prepared and analysed on the same day, because of the reported reactivity of Ag NPs (McMahon et al. 2005).

Prior to spICP-MS analysis, the food additives were dispersed according to a slightly modified version of the above-described method: an accurately weighed subsample of 0.015 ± 0.002 g of the E174 food additive was used, the BSA solution was prepared freshly on each analysis day, and three independent replicates were prepared. All replicates were sonicated for 16 min (applied energy mean ± σ: 32 ± 2 kJ).

The samples were shaken in a Multi Reax (Heidolph Instruments, Schwabach, Germany) until either the silver layer was completely removed from the chocolate core (silver-coated chocolates), or the whole product was dissolved (pearls). In the case of the silver-coated chocolate, the remaining cores were rinsed with another 5 mL of the 0.05% BSA solution, after which the cores were removed from the dispersion. The dispersions were further processed as the E174 food additive dispersions. Three independent replicates were prepared per food product sample. Sampling effects and sample preparation induced artefacts, caused by physical and chemical treatments including probe sonication and choice of dispersion medium, were evaluated. To assess the effect of sonication energy on the particle size and shape, a range of sonication energies (7, 23, 36 and 58 kJ) was applied on product Ag-P-009. In total two repetitions of the experiment were conducted on two different days, based on the generic NANOGENOTOX dispersion protocol described above (Jensen et al. 2011). On each day, one dispersion was prepared. From each dispersion, four TEM specimens (grids) were prepared after sonication. Ag-P-009 was selected because preliminary qualitative TEM analysis showed that it contained a relatively high concentration of particles, allowing size and shape measurement of the constituent particles by quantitative TEM analysis. A non-sonicated dispersion of Ag-P-009 was used as a control. In addition to the TEM analyses of product Ag-P-009, assessing the effect of sonication energy at 7 kJ, 23, 36 and 58 kJ on the particle size and shape, spICP-MS analysis was performed on dispersions prepared according to the same procedure as applied for TEM. Two replicates were thereby analysed on one day. To examine dispersion medium-dependent artefacts by qualitative TEM analysis, dispersions of the pristine food additive Ag-005 were prepared at a concentration of 2.56 mg/mL in pure acetone, a solution of pure acetone and sodium chloride (1:1), and a polyvinylpyrrolidone (PVP, 40,000 g/mol, Sigma-Aldrich, Steinheim, Germany) solution at concentrations of 0.03, 0.1, 0.5 and 3 mM, based on the studies on bulk silver of Huang et al. (2014) and Dang et al. (2012). As a control, dispersions were prepared in distilled water and in 0.05% w/v sterile-filtered BSA solution, combined with 0.5% v/v ethanol pre-wetting. TEM specimens (grids) were prepared from dispersions after a reaction time of 0 and 1 h.
taking into account that the specimen preparation takes approximately 15 min.

**Physico-chemical characterisation by EM**

**TEM specimen preparation**

TEM specimens (grids) were prepared using Alcian blue treated, positively charged pioloform- and carbon-coated, 400 mesh copper grids (Agar Scientific, Stansted, Essex, UK), by drop deposition based on the SOP ‘Preparation of EM-grids containing a representative sample of a dispersed nanomaterial’ (NANoREG 2017). For products and food additives in which low amounts of particles were detected after qualitative inspection of TEM specimens prepared by the former method, silver flakes were collected on an EM grid as described by Verleysen et al. (2015). Filter paper was used for drying the grids from beneath, avoiding removal of the silver material from the grid.

**TEM imaging**

TEM imaging was performed using a Tecnai G2 Spirit TEM with BioTwin lens configuration (Thermo Fisher Scientific, Eindhoven, The Netherlands), equipped with a bottom-mounted 4 x 4 K Eagle CCD-camera (Thermo Fisher Scientific). Micrographs were recorded using the TEM imaging and analysis (TIA) software (Version 3.2, Thermo Fisher Scientific), and converted to TIFF-format, following the SOP ‘Transmission electron microscopic imaging of nanomaterials’ (NANoREG 2017). The SOP foresees that the micrographs are randomly and systematically recorded at 10 positions, pre-defined by the microscope stage and evenly distributed over the entire grid area, to avoid subjectivity in the selection of particles by the analyst. For each material, a set of calibrated micrographs that represent the material on the TEM specimen was recorded. Based on the descriptive TEM analysis, magnifications in the range 30,000 to 48,000 times with a working range covering the complete size distributions, were selected for quantitative TEM analysis. The lower limit of quantification (LLOQ) (between 2.2 nm and 3.8 nm) and upper limit of quantification (ULOQ) (between 91.7 nm and 153.7 nm) were determined supporting on Merkus (Merkus 2009) and ISO 13322–1 (ISO 2004), respectively.

**Electron diffraction**

The crystallographic structure of all materials was monitored by electron diffraction using a Tecnai G2 Spirit TEM with BioTwin lens configuration (Thermo Fisher Scientific, Eindhoven, The Netherlands), equipped with a bottom-mounted 4 x 4 K Eagle CCD-camera (Thermo Fisher Scientific) to evaluate the stability of silver during the analysis. Diffraction patterns of regions containing large flakes and/or particles were recorded, indexed and compared to a database (Inorganic Materials Database (AtomWork) 2010).

**Energy-dispersive X-ray spectroscopy**

TEM specimens (grids) were mounted on a stub and analysed by scanning electron microscopy (SEM) and EDX using a JEOL JSM–7800 F Field Emission SEM operating at 20 kV. Representative SEM images were recorded in Gentle Beam mode at a working distance of 2.8 mm using the software PCSEM. For EDX analysis, a X-MaxN Silicon Drift Detector with a detector size of 80 mm² active area and AZTe® EDS software NanoAnalysis (Oxford Instruments, High Wycombe, UK) was used. In addition, scanning transmission electron microscopy (STEM) and EDX analyses were performed on selected samples to evaluate the stability of silver during the analysis. The STEM-EDX instrumentation is described in the supplementary information (S2).

**Quantitative TEM analysis**

Physical particle properties were measured by quantitative TEM analysis as described by Verleysen et al. (2019). The ParticleSizer software was applied to measure the size and shape of the particles, based on the SOP ‘Measurement of the minimal external dimension of the constituent particles of particulate materials from TEM images by the NanoDefine ParticleSizer software’ (NanoDefine 2016; Wagner 2016; Mech et al. 2020). A region of interest was selected on all images. Rolling ball radius, smoothing factor and variable minimal object-to-background threshold settings were optimised to reduce noise and subtract the background. Particles on the border of the region of interest, and structures with an aberrant morphology, judged to be artefacts based on visual inspection, were omitted from analysis.
Images were recorded and analysed until at least one hundred particles were identified and measured, allowing reliable determination of the particle size distributions of the Ag NPs, as determined based on the validation study of representative test material NM-300K (ICEN European committee for standardization 2018). In this study, the intermediate precision only changed marginally when the number of particles was higher than one hundred. The raw data were processed using an in-house python script for calculation of descriptive statistics, making histograms and curve fitting. For all materials, distributions of the minimal Feret diameter (Feret min), the maximal Feret diameter (Feret max), the equivalent circular diameter (ECD), the aspect ratio, the solidity and the sphericity were determined. Particle perimeter and area were used to calculate the values of the sphericity. The measurement uncertainties were estimated based on recent studies of Verleyesen et al. (2019) and Waegeneers et al. (2019). The measurement uncertainty on the median Feret min of particles present in food additives and products was derived by the quadratic summation of the uncertainty derived for the representative test material NM-300 K (5.9%; k = 1) with the uncertainty on sample preparation (6.8%; k = 1), based on Waegeneers et al. (2019). This results in an expanded measurement uncertainty (k = 2) of 18% on the median Feret min. Assuming that the sample preparation uncertainty can be applied as well to other size, shape and surface structure parameters, determined from the same data sets, the expanded measurement uncertainty on the ECD, aspect ratio and solidity are 18%, 15% and 15%, respectively.

**Chemical characterisation by ICP-OES**

Total silver analysis was limited to the products containing E174. Prior to total silver analysis, the samples were homogenised by means of a laboratory knife mill (IKA A11 basic) after freezing the samples shortly at −80°C to make them more brittle. The total silver content in the products was quantified by ICP-OES (Varian 720-ES, Mulgrave, Australia) in three independent replicates per product. An accurately weighted sample of ± 0.25 g homogenised product was brought in a Teflon digestion vessel to which 6.00 mL of nitric acid (HNO₃ 67–69% SpA grade, Romil, Cambridge, UK) and 2.00 mL sulphuric acid (H₂SO₄ 93–98% SpA grade, Romil, Cambridge, UK) were added. The samples were digested in a MARS XPRESS microwave system (CEM Corporation, Matthews, NC, USA) operated at 1280 W power. The applied temperature program went from room temperature to 180°C in 20 min followed by a constant temperature of 180°C for 30 min. After cooling down to room temperature, the extracts were diluted with double-distilled water to obtain a total dilution of the sample of 1:700 (w: w). Silver was measured at wavelength 328.068 nm. Wavelengths 338.289 and 241.318 nm were monitored as well to verify the absence of interferences. Calibration was performed within each sample by

**Physico-chemical characterisation by spICP-MS**

All dispersions were vortex stirred for 30 s prior to dilution for spICP-MS analysis. Each dispersion was diluted in polypropylene vials to two different levels with ultrapure water (UPW, 18.2 MΩ/cm), which was prepared by using a Millipore Integral 3 system (Millipore, Molsheim, France). The two appropriate dilutions were determined after a range-finding test, during which different dilutions of the dispersion were measured. The two dilutions had to result in a proportionally changing number of detected particles, a constant particle size, and 200–2200 detected particles within the 1-min acquisition time. All vials and polypropylene tubes were washed with acid prior to use. The dispersions were analysed by spICP-MS as described by Waegeneers et al. (2019) to obtain distributions of the equivalent spherical diameter (ESD), particle number concentrations (Cₚ), and particle mass concentration (Cₘ). The method was validated and measurement uncertainties were determined for food additives and products (Waegeneers et al. 2019). spICP-MS analysis was performed on the whole product in case of products without chocolate (pearls) or on part of the product, i.e. after removal of the chocolate core, in case of silver-coated chocolates. The latter results were, however, recalculated and expressed on the whole product as well. The applied spICP-MS instrumentation is described in the supplementary information (S3).
means of standard addition. The determination of the measurement uncertainty on the silver analyses is described in the supplementary data (S4).

Results

Qualitative description based on TEM imaging

The positive control Ag-001 was homogeneously distributed over the complete grid surface and consisted of near-spherical nanoparticles (Figure 1i-l). In all food additives (Figure 1a-d) and products (Figure 1e-h), both (nano)particles and micrometre-sized flakes were observed on the TEM images. The distribution of particles was not uniform over the grid surface. Most particles were found in the proximity of the micrometre-sized flakes. The particles had either an irregular or a near-spherical shape, and showed diffraction contrast. TEM analysis showed lower amounts of particles in the food additives than in the products (Table S3). The surface structure of the micrometre-sized flakes was smoother and more uniform in the food additives than in the products (Figure 1).

TEM sampling effects and sample preparation

Sampling of particles on TEM specimens

All products dispersed according to Verleysen et al. (2015) resulted in a sufficient number of particles on the grid for quantitative TEM analysis (Table 1). For food additives, dispersing the particles was difficult and a limitation for representative sampling. For half of the food additives, a number-based particle size distribution could reliably be determined by quantitative TEM analysis. For four food additives, Ag-008, Ag-009, Ag-010 and Ag-011, both sonicated and non-sonicated dispersions resulted in a sufficient number of particles on the grid for quantitative analysis (Table 1). Four other food additives (Ag-004, Ag-005, Ag-006, Ag-007) required sonication to sufficiently increase the number of particles on the EM grids, which was close to the minimum amount (>100 particles) needed to allow reliable quantitative TEM analysis (Table 1). For Ag-002, sonication reduced the number of particles, possibly due to their sonication-induced interaction with the food additives potassium sorbate (E202) and acacia gum (E414) present in this sample. Therefore, Ag-002, which showed a high enough number of silver particles, was measured without an additional sonication step. For Ag-003, sonication did not result in an increase in the number of particles on the grid. Even though their concentration was low, the same types of particles were observed in the food additives regardless whether sonication was applied or not.

For spICP-MS it was essential to apply the NANOGENOTOX protocol (Jensen et al. 2011) with sonication to homogenise and disperse the samples, and measure a sufficient number of particles. For two materials (Ag-002 and Ag-006), the particle number remained too low to determine a size distribution. For spICP-MS, the criterion was thereby the detection of minimally 200 particles within the 1-min acquisition time in at least one out of three replicates.

Effect of sonication

TEM analysis showed that increasing the sonication energy broke up the larger micrometre-sized flakes into smaller micrometre-sized flakes (Figure S1). Also, an increased number of isolated nanoparticles was observed, suggesting gradual release of particles from the surface of the flakes (Figure 2). Assuming that nanoparticles generated from the breakdown of flakes by sonication are irregular, the near-spherical morphology of most of these nanoparticles suggested they were detached off the micrometre-sized flakes.

Both TEM and spICP-MS size measurements showed that the size of the constituent particles remained relatively constant over the range of sonication energies, within the reported uncertainty intervals (Figure 2). Even so, for TEM, a minor decrease in the median Feret max, was observed between 0 kJ and 7 kJ sonicated samples on the one hand, and 23, 36 and 58 kJ sonicated samples on the other hand (Figure 2). The lower Feret max (Table S4) for sonication energies higher than 7 kJ suggest a de-agglomeration effect. Sonication-induced breakdown of the constituent particles is unlikely, since the Feret max remained constant with further increasing sonication energies up to 58 kJ. This assumption is corroborated by the increasing trend in particle number concentration with increasing
sonication energies above 7 kJ observed by spICP-MS (Figure 2). An overview of the spICP-MS results is given in Table S5. The large flakes and low amount of individual particles in the non-treated sample (0 kJ) made the determination of the particle size distribution by spICP-MS impossible.

**Effect of dispersion media**

In the dispersions prepared in acetone, Ag NPs were found with rod-like to cubic shapes, different from the observed Ag NPs in the non-acetone-treated control samples. This effect was enhanced in alkaline medium and when incubation was prolonged to 2 h (data not shown). Ag NPs with aberrant shapes were also detected when a PVP-containing dispersion medium was tested (data not shown). These particles were observed immediately after specimen preparation corresponding with a reaction time of approximately 15 min. After prolonged exposure (1 h) of E174 particles to a series of PVP concentrations (0.5, 1 and 3 mM), an increased number of aberrant particles was observed.

Particles with specific rod-like to cubic shapes were not observed in the control samples, in any of the 10 food additives dispersed in 0.05% w/v sterile-filtered BSA solution, or in any of the 10 products prepared using only distilled water. The near-spherical or irregular shape of the particles in the control samples, food additives and products suggests that the selected sample preparation protocols did not result in medium-induced particle deformation or particle generation.

**Characterisation of silver particles in E174**

**Identification and stability**

EDX confirmed that the majority of particles and flakes present in the food additives and products consist of silver because no other specific signal than silver collocated with the silver signal (Figure 1b-c; f-g). Identification of individual nanoparticles smaller than about 10 nm by SEM-EDX was difficult due to the detection limit (Carter and Williams 2009; Scimeca et al. 2018). In these cases, STEM-EDX analysis of selected samples (Table S2) confirmed that
these smaller particles also consisted of silver. Silver particles detected by STEM-EDX showed no coinciding sulphur signal at the particle surface, indicating that no transformation to silver sulphide occurred during the analysis time. McMahon et al. (2005) report such a transformation after increasing exposure of Ag NPs to ambient laboratory air. Copper, carbon, oxygen, and silicon background signals were often detected and originate, among others, from the carbon and pioloform-coated copper EM grid and from the microscope and EDX detectors. Other components detected by EDX in the products are given in Table S2.

Electron diffraction analysis of regions containing both micrometre-sized flakes and nanoparticles showed no phase transition of silver during the time needed for the analysis (Figure S2-3). Since very few particles were detected by TEM, the majority of the signal in the diffraction pattern originated from the micrometre-sized flakes.
**Size, shape and surface structure measurements**

The ParticleSizer software succeeded in applying noise reduction and background subtraction, allowing robust automatic thresholding based on mass-thickness and diffraction contrast, and reliable detection and measurement of the large majority of particles, identified as silver by EDX.

In food additives, the median Feret min of the particles measured by quantitative TEM analysis ranged from 5 to 20 nm (Table 1, Figure 3), with an overall mean of 11 ± 4 nm. The expanded measurement uncertainties are presented as error bars in Figure 3, and are given in Table 1 together with the quantitative results. The low amount of particles (<10 particles per image) detected on the grid (Table S3) made quantitative TEM analysis challenging. Micrometre-sized flakes were not accounted for in the number-based size distribution, because they are much larger than the ULOQ for the applied imaging conditions (approximately 91.7–153.7 nm). As the large particles (micrometre-sized and larger) were low in number compared to the nanosized particles, they had a negligible effect on the number-based distributions. The median ESD determined by spICP-MS ranged from 14 nm to 27 nm (Table 1, Figure 3), with an overall mean of 19 ± 4 nm. The food additives showed a high background signal, originating from ions and/or multiple particles below the LLOQ, combined with a relatively low number of nanoparticles, making the particle detection and quantification by spICP-MS difficult.

In products, the number of particles was high enough reliably to determine the particle size distributions by both quantitative TEM analysis and spICP-MS. The median Feret min determined by TEM ranged from 9 to 36 nm (Table 1, Figure 3), with an overall mean of 18 ± 7 nm. The median ESD determined by spICP-MS ranged from 16 to 24 nm, with an overall mean of 21 ± 2 nm (Table 1, Figure 3).

The LLOQ of the spICP-MS size measurement (11–20 nm, depending on the sample) was higher than that of quantitative TEM analysis (approximately 2.2 nm to 3.8 nm). The median ESD measured by spICP-MS was higher than the Feret min measured by TEM partly because it is a different size parameter and partly because particles below the LLOQ of spICP-MS are not detected, which shifts the median towards higher values. Above the LLOQ of spICP-MS, the number-based size distributions determined by TEM and spICP-MS agreed well (Figure 4a-c).

Similar median values of the aspect ratio and solidity were obtained for particles in products and food additives and for the control particles (Ag-001). Median aspect ratios ranged from 1.07 to 1.42 and solidities from 0.91 to 0.99. The majority of particles have a high sphericity, defined according to Krumbein and Sloss (1963).

**Concentration**

Single-particle ICP-MS allowed to measure the particle mass and number concentrations of the particles with an ESD between the LLOQ and 100 nm. In food additives, the particle mass concentrations ($C_m$) varied between 1.7 and 5.5 g/kg, while the number concentrations ($C_p$) varied between 0.7 and $3.5 \times 10^{16}$ particles/kg. In products, the particle mass concentrations varied between 0.003 and 0.03 g/kg (expressed on the whole product). The number concentrations varied between 0.17 and $1.6 \times 10^{14}$ particles/kg (expressed on the whole product). The higher background signal and the lower particle number in food additives made the setting of the detection threshold more difficult in the food additives compared to the products. Therefore, the quantification of particles by spICP-MS in the food additives was less reliable than in the products.

The total silver concentration in the products ranged from 1.4 to 8.7 g/kg product and was used to estimate the mass percentage of the particles smaller than 100 nm in these products. The average mass percentage was 0.32% (range: 0.16–0.53%) (Table 1). The uncertainties on these mass percentages may be high as only 50% of the theoretical Ag NP mass could be recovered by spICP-MS in a representative test material (Waegeneers et al. 2019). In food additives, the total silver concentration was not determined by ICP-OES, because silver was, besides some impurities (Table S2), the sole ingredient (except for Ag-002). The mass percentages of nano-sized particles were estimated assuming a total silver concentration of 1000 g/kg and was on average 0.30% (range 0.17–0.55%) (Table 1).
**Figure 3.** Median values of the Feret min (TEM) and the ESD (spICP-MS) obtained for each food additive or product. The error bars represent the confidence interval based on the expanded combined uncertainty ($U_{ex}$) for the measurement of the median Feret min and the ESD.

**Figure 4.** Comparison of the number-based distributions obtained by TEM and spICP-MS analyses of (a) Ag-011, (b) Ag-P-009 (c) Ag-001.
For all materials, independently of the choice of technique, the nano-sized particles represent more than 97% (by number) of the silver particles, even though the largest amount of silver (by mass) is present in the flakes. Although the variation in the number of particles per TEM micrograph was high, three times more particles were observed per surface area in preparations of products than of food additives, indicating higher numbers of nanoparticles in products than in food additives (Table S3).

Discussion

This work demonstrates the presence of a significant amount of Ag NPs in pristine E174 food additives and E174-containing products by TEM and spICP-MS. Ninety seven per cent or more of the silver constituent particles (by number) measured by TEM in the examined food additives and products were smaller than 100 nm. The physico-chemical characterisation results classify E174 as a nanomaterial according to the EC recommended definition (EC 2011).

The broad selection of E174-containing samples, comprising silver leaves, petals, powders, silver-coated pearls and sugar beans results in more robust physico-chemical characterisation compared to the limited number of analysed samples reported earlier (Verleysen et al. 2015; de la Calle et al. 2018). In addition, the presented approach applied standardised and validated methods. As opposed to earlier characterisations of Ag NPs in E174 (Verleysen et al. 2015; de la Calle et al. 2018), the reported uncertainties, include the uncertainty associated to sample preparation (Waegeneers et al. 2019), which for the measured food additives and products accounts for approximately half of the expanded measurement uncertainty on the size (Feret min) measurement (in the order of 18%).

The difficulties for TEM quantification associated with sampling of low numbers of particles were partly overcome by sonication. Sonication was indispensable for the analysis of particles in the food additives Ag-004, Ag-005, Ag-006, Ag-007 by TEM, and in food additives and products by spICP-MS. However, the applied NANOGENOTOX (Jensen et al. 2011) dispersion protocol required monitoring of artefacts, because of the reported transformations of nano-silver, such as the release of silver ions and aggregation (Ahlberg et al. 2014; Vazquez-Muñoz et al. 2017). The tested range of sonication energies (7, 23, 36 and 58 kJ), and the selected medium (0.05% w/v sterile-filtered BSA solution), did not induce any measurable effects on the size of the particles as observed by TEM and spICP-MS, supporting the validity of the selected sample preparation methodologies. Dispersion protocols based on chemical treatment with PVP and acetone were shown to be unsuitable to improve the dispersion of the particles, because of the de novo formation of Ag NPs, probably by reduction of silver ions by a reducing medium as reported earlier by Gomes et al. (2015), Koczkur et al. (2015) and Tsuji et al. (2017).

Size, shape, chemical composition, phase and surface characteristics of the Ag (nano)particles were characterised in line with EFSA’s Guidance on risk assessment of application of nanoscience and nanotechnology, which requires at least two independent techniques to characterise the NPs present in foods ([EFSA] European Food Safety Authority 2018). The combination of TEM, S(T)EM-EDX, electron diffraction, spICP-MS and ICP-OES allowed a thorough characterisation of both the pristine food additives and the products. The measured size, shape, and surface topology distributions are in line with earlier reported characterisation results of constituent particles in silver-coated confectionery (Verleysen et al. 2015; de la Calle et al. 2018).

Single-particle ICP-MS was successfully applied to determine the mass and number concentrations of nano-sized particles that were larger than the LLOQ in E174 confectionery. These concentrations ranged, respectively, from 3 ± 1 to 28 ± 9 mg/kg and 0.17 ± 0.06 to 1.6 ± 0.5 x 10^{14} particles/kg and are in line with earlier studies on silver-coated confectionery (Verleysen et al. 2015; de la Calle et al. 2018). Because standardised and validated methodologies were applied, the mass in confectionery which consumers are exposed to, produced here, are more representative than the values reported earlier by our laboratory (Verleysen et al. 2015), measuring respectively 1.8 ± 0.6 mg/kg and 4.4 ± 0.5 x 10^{12} particles/kg. The total silver concentrations measured in the products, which were in the g/kg range, corresponded to the use level of chocolate coatings reported by EFSA ([EFSA] European Food Safety Authority 2016). On average,
0.30% (by mass) of the silver in the products was present as nanoparticles in contrast to the 20% found previously (Verleysen et al. 2015). The difference can be explained by an incomplete extraction of the total Ag measured in the latter study of only 0.008 g/kg, which is far below the Ag concentrations measured here. The method applied in Verleysen et al. (2015), which was appropriate for the DOLT-4 certified reference material matrix (dogfish liver), was insufficient to either extract all silver or keep it in solution for the confectionery samples.

Interfering signals, originating from ions and/or multiple particles below the LLOQ detected within a dwell time, combined with a relatively low number of nanoparticles made the particle characterisation of food additives by spICP-MS challenging, as this resulted in difficult thresholding. Applying larger dilution factors to reduce this background signal often resulted in a too low number of detectable particle signals within the 1-min acquisition time. By consequence, the clear distinction in particle numbers between the food additives and products observed by TEM was not corroborated by the spICP-MS results when expressing the particle number concentrations per unit of total silver mass. Erroneous classification of some interfering signals as particles, if the threshold was set too low, might partly explain the higher number of detected particles observed in food. Such interference does not occur in TEM measurements. Tuoriniemi et al. (2012) demonstrated that larger thresholds are necessary for smaller particles to reduce the number of false positives. On the other hand, the larger the threshold, the more true particle signals are removed. A signal threshold level of mean + 5σ was chosen here as a compromise, but it does not exclude that an overestimation due to false positives as well as an underestimation due to removing of particles, might have occurred in the food additives. Since TEM is not suited for measuring particle concentrations, more research is needed to explain the apparently different results for particle numbers found in food additives and products determined by TEM and spICP-MS.

Previous risk assessment studies on E174 mainly focused on silver in its bulk, ionic or pristine nano-form ([EFSA] European Food Safety Authority 2016), while this study also considered food matrix-induced alterations to the fraction of Ag NPs in E174. The higher concentration of particles detected on the EM grids and the rougher structure of micrometre-sized silver flakes in the products compared to the food additives may suggest a production process-induced effect. A relation between surface deformations of silver foils and pre-treatments was established in earlier work (Liu et al. 2010), and may explain this effect. Liu et al. found that exposure of macroscopic silver foils to surface-active agents, pH change or air-saturated environments lead to destabilisation of their surface. By SEM, a transformation of a smooth surface to irregular nodules was shown similar to our observations for silver in food products. This is an important factor for the risk assessment of E174 and implies that the risk assessment should not only consider the pristine food additive but also the E174 nanoforms in products. Martirosyan and Schneider (2014) have previously stressed the importance of studying the relationships between particle characteristics, food pH/polarity and environmental conditions relevant to food production, storage and packaging. Due to absence of a coating, the detected Ag NPs are a potential source of Ag⁺ ions which are released more rapidly from the particle surface compared to coated Ag NPs (Liu et al. 2010). Surface and shape characteristics of the constituent particles, assessed, respectively, as the solidity, the aspect ratio and sphericity, were relatively consistent for the analysed samples. These properties can serve to further predict silver’s dissolution to Ag⁺, further fate, and in vivo and in vitro toxicity. Future toxicity studies can be designed based on Ag NPs with similar characteristics, as found in this study, to better estimate the nano-hazard related to the use of E174 or silver-containing formulations.

In summary, silver nanoparticles were detected in 10 commercially available food additives and 10 food products containing E174. A higher concentration of particles and a rougher structure of the micrometre-sized silver flakes in the products compared to the food additives are observed by TEM. This is an important factor for risk assessment and implies that risk assessment should not only consider the pristine food additive but also the E174 nanoforms in products. Further investigations are
needed to explain the difference in particle numbers between products and food additives observed by TEM. The results of this study addressed, at least partly, the knowledge gaps indicated by the EFSA Panel on Food Additives (ANS Panel) in its scientific opinion on the re-evaluation of silver as food additive, such as unknown particle size distributions and the lack of relevant data for toxicity studies ([EFSA] European Food Safety Authority 2016). The approach can be used to examine the stability, transformations and uptake of silver nanoparticles to support in vitro and in vivo toxicity testing.

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