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Physicochemical characterization of nanoparticles in food additives in the context of risk identification

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Abstract

The joint Nanofood@ - EFSAnano project developed analytical methodologies for identification and characterization of nanoparticles in food additives. The methodologies were applied in a regulatory context for control and risk identification purposes. In specific, the project developed methods to characterize E 171 (titanium dioxide), E 174 (silver) and E 175 (gold) food additives in their pristine state and in the food matrix. The project focused on method development based on TEM and (sp)ICP-MS, method standardization and validation, and application of the methods in a wider scope for market surveillance. In addition, a pristine E 171 intra-laboratory reference material was produced; 300 homogeneous and stable vials were fractionated and a homogeneity study was performed. The methodologies, analysis results and the developed expertise form a strong base to fulfil control activities and to provide expertise in the characterization of materials which may contain a fraction of nanoparticles, applied in the food chain. They can be applied to implement the "EFSA guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles", and the "EFSA guidance on the human and animal risk assessment of the application of nanoscience and nanotechnologies in agri/food/feed".

Key words: food additives E 171, E 174, E 175; electron microscopy; single particle ICP-MS; physicochemical characterization; nanoparticles, particle size distribution

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Summary

The joint nanofood@ - EFSAnano project developed expertise in the characterization of materials which may contain a fraction of nanoparticles, applied in the food chain. The project focused on delivering standardized and validated methods to characterize E 171 (titanium dioxide), E 174 (silver) and E 175 (gold) food additives in their pristine state and in food products.

Method development

An approach for the screening, detection and physicochemical characterization of nanoparticles in E 171, E 174 and E 175 food additives was developed. This approach was based on the "Nanotechnologies – Guidance on detection and identification of nano-objects in complex matrices", developed in the context of CEN/TC 352 (CEN, 2018). It combines measuring the particle size distribution with chemical composition analysis, allowing analysis of food additives in their pristine state and in food products. The proposed methodologies include physical characterization by electron microscopy-based techniques, such as transmission electron microscopy (TEM), scanning electron microscopy (SEM) and high angle annular dark field - scanning transmission electron microscopy (HAADF-STEM), and chemical characterization by spectroscopic techniques, such as energy dispersive X-ray spectroscopy (EDX), inductively coupled plasma mass spectroscopy (ICP-MS), inductively coupled plasma - optical emission spectrometry (ICP-OES) and single particle inductively coupled plasma mass spectroscopy (spICP-MS).

A complete analysis includes following steps:

- Sample preparation optimization for EM and ICP-techniques
- Screening for the presence of particles in food additives and food products by descriptive EM and/or spICP-MS
- Confirmation of the chemical identity of the particles by electron diffraction, EDX and spICP-MS
- Measurement of the size –and shape distributions of the particles by quantitative EM
- Determination of the concentration of the fraction of nanoparticles by ICP-MS and spICP-MS.

The developed methods were standardized as standard operating procedures (SOPs), and validated. With material-dependent modifications, the methods can be applied to characterize the fraction of nanoparticles in other food additives.

Physicochemical characterization of nanoparticles in pristine E 174 and food products containing E 174

The results of the analyses performed on E 174 are summarized in the publications of Waegeneers et al. (Waegeneers et al., 2019) and De Vos et al. (De Vos et al., 2020). TEM analysis revealed that all pristine E 174 samples and E174-containing products contained both silver nanoparticles and µm-sized silver flakes. The chemical composition and crystallographic structure of the particles and flakes were confirmed to be silver by SEM-EDX, STEM-EDX and electron diffraction, respectively. Number-based particle size distributions were obtained by quantitative TEM for all E174-containing products and for half of the pristine E174 samples. In the other pristine samples, the amount of particles was too low to obtain reliable number-based size distributions. The median of the minimum external dimension, assessed as minimum Feret diameter (Fmin), of the fraction of particles determined by quantitative TEM analysis was 11 ± 4 nm and 18 ± 7 nm (overall mean ± standard deviation), for pristine E174 samples and E174-containing products, respectively. In general, 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 particles determined by spICP-MS was 19 ± 4 nm and 21 ± 2 nm (overall mean ± standard deviation), for pristine E174 samples and E174-containing 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."

Physicochemical characterization of nanoparticles in pristine E 175 and food products containing E 175

The methodology for the screening, detection and physicochemical characterization of nanoparticles in E 174 was applied for the screening of pristine E 175 samples and E175-containing products. In conclusion, there were no gold nanoparticles detected in pristine E 175 samples and E175-containing products.

Physicochemical characterization of nanoparticles in pristine E 171 and food products containing E 171
To measure the minimum external dimension of the constituent particles of E 171, sample preparation factors influencing particle dispersion (pH, medium, probe sonication and centrifugation) were tested and optimized on 15 pristine E 171 samples, including 9 E171 samples available on the public market and 6 E 171 samples obtained from business operators (Verleysen et al., 2020). Electron diffraction demonstrated that both anatase and rutile TiO_2 particles are sold as E 171, including 3 samples containing smaller rutile TiO_2 particles (20-40 nm) coated with mica, which are potassium aluminium silicate-based pearlescent pigments (EFSA FAF Panel (Panel on Food Additives and Flavourings), 2020).[1]
In optimized conditions, all E 171 samples were shown to contain an important fraction of nanoparticles. The large majority of constituent particles, confirmed to be TiO_2 by EDX, were reliably detected and measured using the applied ParticleSizer software. In the most dispersed state, the particle size distributions obtained by TEM and spICP-MS agreed well. The measurement uncertainty budgets of particle sizing by TEM and spICP-MS were in the order of 10% and 16 % (Ucx, k=2), respectively, based on validation studies with a series of representative test materials (RTM). Using the optimized sample preparation protocols and image analysis settings, 12 of the 15 samples showed a median minimum external dimension below 100 nm. When the expanded measurement uncertainties (Ucx, k = 2) were considered, 11 samples had a median minimum external dimension significantly smaller than 100 nm (median Fmin + Ucx (k = 2) < 100 nm).
In the E 171 samples from the business operators, 18% to 70% of the measured constituent particles were smaller than 100 nm. In the E 171 samples purchased on the public market, excluded the three samples containing potassium aluminium silicate-based pearlescent pigments, 64% to 73% of the measured constituent particles were smaller than 100 nm. TiO_2 particle concentrations estimated by spICP-MS were in the range of 10^{17} particles per kg TiO_2.

A pristine E 171 intra-laboratory reference material was produced and the homogeneity was demonstrated. This E 171 reference material was applied in an inter-laboratory comparison study for spICP-MS among seven experienced European food control and food research laboratories, organized by JRC in the context of the "Nano in Food" project (Geiss et al., 2021).

It was demonstrated that the methodology developed to characterize pristine E 171 can be applied to characterize the particles isolated from products containing E 171, provided that the EM grids contain a sufficient number of constituent particles for which the degree of agglomeration is minimal. Other requirements are that the particles are homogeneously distributed on the grid and that they are representative for the sample. This generally required optimization of the sample preparation. A generic protocol for sample preparation and physicochemical characterization of food additive E 171 in a food matrix was developed and evaluated on 15 different types of food products, available on the European market (Verleysen et al., in preparation). The protocol combines a generic sample preparation protocol for matrix extraction and representative sampling with total concentration measurement by ICP-MS and particle size, shape and elemental composition measurement by electron microscopy based techniques. The methodology can be adapted for analysing TiO_2 in cosmetics, personal care products and medicines.

[1] 'Potassium aluminium silicate-based pearlescent pigments' are mentioned in the EFSA opinion on the re-evaluation of E555 where it is indicated "Interested business operators stated that 'potassium aluminium silicate-based pearlescent pigments' (e.g. potassium aluminium silicate coated with titanium dioxide) used on a food product is labelled as E 171 or titanium dioxide" and the EFSA FAF Panel concluded that "potassium aluminium silicate in 'potassium aluminium silicate-based pearlescent pigments' does not meet the definition of a carrier according to Regulation (EC) 1333/2008 and 'potassium aluminium silicate-based pearlescent pigments' are not listed in Regulation (EC) 1333/2008. The Panel also concluded that 'potassium aluminium silicate-based pearlescent pigments' should be evaluated as a new food additive" (EFSA FAF Panel (Panel on Food Additives and Flavourings), 2020)

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1. Introduction

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

To identify and characterize nanoparticles in food additives in a regulatory framework, a major challenge is to develop analytical methodologies which allow analysing different types of food additives in their pristine state and in the food matrix. Such analytical methodologies should combine a high spatial resolution to measure particles down to 1 nm, with enough sensitivity to identify particles e.g. based on chemical composition. In addition, there is a need for characterization of nanoparticles in the context of risk identification, with a focus on the metrological sound validation of the developed and applied methods. In this context, the joint Nanofood@ - EFSAnano project delivered standardized and validated methods to characterize E 171, E 174, and E 175 food additives in their pristine state and in food products with a known measurement uncertainty.

The first part of the project (WP1-WP4) entitled "Implementation and validation of an analytical methodology to assess nanomaterials in food additives" (acronym: Nanofood@), was supported by the service Contractual Research of the Belgian federal public service (FPS) Public Health. The project activities are shown in blue boxes in Figure 1.
The Nanofood@ project focused on method development and aimed to answer following specific research questions:

- Do E 171, E 174 and E 175 food additives contain a fraction of nanoparticles (WP1)?
- What is the crystallographic structure of these food additives and what is the elemental composition of the individual particles (WP2)?
- Can the size distribution of the particles in these food additives be determined using number based methods, in line with the definition of a nanomaterial?[2] What are the uncertainties associated with this measurement (WP3)?
- Can the concentration of silver, gold and titanium dioxide nanoparticles in food additives be determined accurately and precisely (WP4)?

The approach followed in WP1 – WP4 to characterize food additives was based on a combination of physical characterization by electron microscopy imaging - based techniques, such as TEM, SEM and STEM, combined with chemical characterization by spectroscopic techniques, such as S(T)EM-EDX, ICP-MS and spICP-MS, and supported on the "Nanotechnologies – Guidance on detection and identification of nano-objects in complex matrices", developed in the context of CEN/TC 352 (CEN, 2018).

The Nanofood@ project was complemented by research activities sponsored by DG4 of the FPS Public Health (the NanoAg@ project) and EFSA (EFSAnano -GP/EFSA/AFSCO/2017/06) (WP5-WP9). The latter activities are shown in the green boxes in Figure 1.
The EFSAnano project aimed to answer following specific research questions:

- Can the approach to analyse the nanoparticles in food additives E 171, E 174 and E 175 in their pristine state be adapted to analyse listed food additives in a food matrix, e.g. by adaptation of sample preparation?
- Do the results and methods fill the knowledge gaps identified during the re-evaluation of the listed food additives (EFSA ANS Panel 2016a,b,c)? Will they allow to contribute to support the follow-up of the re-evaluation of the listed food additives?
- Can this expertise be used to develop general guidelines for screening, systematic detection and characterization of nanoparticles in food additives in the pristine state and in a food matrix? Can the methodologies be validated in a wider scope?
- What is the exposure of consumers to the fraction of nanoparticles of selected food additives?

To answer these questions, the EFSAnano project implemented the methodologies developed in WP1-WP4 in a systematic and larger scale examination of food additives in their pristine state and in food products available on the market, in order to obtain results essential for risk identification. More specific,

[2] A number-based method provides a number-based size distribution of the material presenting the size distribution based on the number concentration (i.e. the number of objects within a given size range divided by the number of objects in total) and not on the mass fraction of nanoscale particles in the material as a small mass fraction may contain the largest number of particles."

the project focused on developing a reference material (WP5), and on applying validated methods to different batches of E 171, E 174, and E 175 food additives in their pristine state and in food products of several producers (WP6-WP7). Based on these elaborated examples, the project aimed to contribute to a generic approach to identify and characterize the fraction of nanoparticles in food additives.

In addition to the physicochemical characterization of the food additives, the chronic dietary exposure of the Belgian population to silver nanoparticles in food additive E 174 was assessed (WP8). The results of this exposure study are not included in this publication, but will be reported separately by Janssens et al. (Janssens et al., in preparation).

To assure a quality of data in line with the requirements of the EC definition of nanomaterials (Commission Recommendation 2011/696/EU[3]) and with EFSA Guidance (EFSA Scientific Committee, 2018), the project aimed at developing harmonized material datasheets (MDS) for reporting number-based size and shape distributions, particle concentrations and associated measurement uncertainties (WP9). Such size information is specifically requested in the Scientific opinions on the re-evaluation of food additives such as E 171 (EFSA ANS Panel (EFSA Panel on Food Additives and Nutrients Sources added to Food), 2016a; EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), 2019), E 174 (EFSA ANS Panel (EFSA Panel on Food Additives and Nutrients Sources added to Food), 2016b) and E 175 (EFSA ANS Panel (EFSA Panel on Food Additives and Nutrients Sources added to Food), 2016c).

A detailed overview of the tasks of this research project is presented in Table 1.

This contract/grant was awarded by EFSA to: Service Trace Elements and Nanomaterials, Sciensano

Contractor/Beneficiary: Service Trace Elements and Nanomaterials, Sciensano

Contract/Grant title: Physicochemical characterization and exposure analysis of nanoparticles in food additives in the context of risk assessment

Contract/Grant number: EFSAnano -GP/EFSA/AFSCO/2017/06

[3] Commission Recommendation of 18 October 2011 on the definition of nanomaterial. OJ L 275, 20.10.2011, p. 38–40.

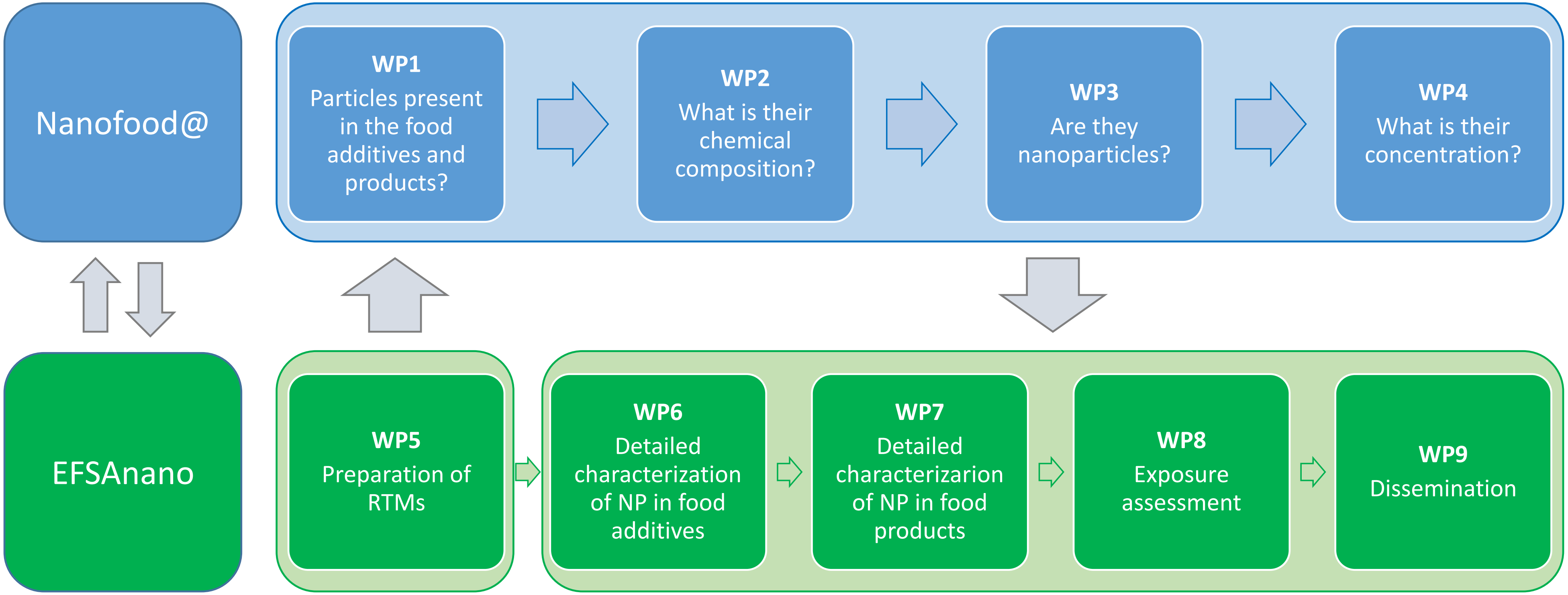

Figure 1 Overview of the approach proposed in this research project showing the key questions and the methodologies for each of the operational work packages of the project. The work packages presented in blue boxes are supported by the Nanofood@ project of Contractual Research (Belgian federal government). The work packages presented in the green boxes are supported by EFSA (GP/EFSA/AFSCO/2017/06) and DG4 (NanoAg@).

Table 1 Overview of the work packages (WP) and tasks. WP 1 to 4, correspond to the Nanofood@ project, WP 5 to 9 correspond to the EFSAnano project.

Nanofood@ project		EFSAnano project	
Code	**Description**	**Code**	**Description**
WP1	**Particles present in the food additives and products?**	**WP5**	**Preparation of representative test materials for the food additives E 171, E 174, E 175**
T1.1	Optimization of sample and specimen preparation for analytical and quantitative EM	T5.1	Purchase of batches of food additives
T1.2	Descriptive TEM analysis	T5.2	Fractionation of the representative test materials
		T5.3	Homogeneity study
WP2	**What is the chemical composition of the particles in the food additives and products?**	**WP6**	**Detailed characterization of nanoparticles in food additives in their pristine state**
T2.1	Crystallographic characterization by electron diffraction	T6.1.	Analysis of pristine samples of E 171
T2.2	Chemical analysis by SEM and EDX	T6.2	Analysis of pristine samples of E 174
T2.3	Chemical analysis by HAADF-STEM and EDX	T6.3	Analysis of pristine samples of E 175
WP3	**Are the particles of the food additives and products "nano"?**	**WP7**	**Detailed characterization of nanoparticles in food products containing the food additives**
T3.1	Quantitative TEM analyses of physical particle properties	T7.1	Analysis of food products containing E 171
T3.2	Quantitative HAADF-STEM analyses of physical particle properties	T7.2	Analysis of food products containing E 174
		T7.3	Analysis of food products containing E 175
WP4	**What is the concentration of the (nano)particles in the food additives and products?**	**WP8**	**Assessment of the exposure of consumers to nanoparticles in selected food additives**
T4.1	Method development and validation of the total mass of the chemical in the food additive containing products using ICP-MS	T8.1	Establishing the food categories and food consumption data to be used for the exposure assessment
T4.2.	Development of sample preparation protocols for analysis by spICP-MS	T8.2	Dietary exposure to selected food additives
T4.3.	Measurement of size and number of the particles using spICP-MS	T8.3	Dietary exposure to nanoparticles contained in the food additives
		WP9	**Dissemination of the expertise and results to competent national and European institutions**
		T9.1	Organisation of dedicated workshop
		T9.2	Transfer of technologies to European network
		T9.3	Transfer of results in SSD format or similar and in peer reviewed publications

2. Materials and Methods

2.1. Materials

E 174 and E 175 are food additives approved by the European Commission to be used for the external coating of confectionery, for decoration of chocolates, and in liqueurs (Regulation (EC) No 1333/2008[4]). Commission Regulation (EU) No 231/2012[5] of 9 March 2012 laying down specifications for food additives authorised in the EU according to Regulation (EC) No 1333/2008 of the European Parliament and of the Council notes that they are generally applied as powder or as tiny sheets.

Originally, food additive E 175 (gold) was chosen because it was judged to be an easily accessible experimental model, with the advantage that gold in bulk form is chemically inert. In general, only nanogold particles in the order of 5 nm and smaller are reported to have altered electrical, chemical and physical properties. Because of its high molecular weight, its stability and its low abundance (background) in nature, it is relatively easy to detect and analyse in a food product by microscopy and spectroscopy based detection methods. In the project, both pristine E 175 samples and food products containing E 175 (Table 2), including liquors, wine confit and syrup, were analysed. E 175 containing products were difficult to find on the market.

E 174 (silver) is generally applied as powder or flakes. Silver is a more difficult model than gold because it is relatively unstable in its nano-form when exposed to air, and tends to transform over time, e.g. into silver sulphide (Ag_2S). Combining elemental analysis by EDX with determination of the crystallographic phase by electron diffraction allows monitoring such transformations.
As a representative test material (RTM) for method development and validation, the silver manufactured nanomaterial NM-300K (Klein et al., 2011) was selected. This material has been extensively measured by TEM and spICP-MS and its characterisation is validated in the Service Trace Elements and Nanomaterials of Sciensano for both methods. In addition, a second silver reference material was obtained from a local pharmacy (Ag-001). This colloidal material was shown to be very similar to NM-300K.
Ten pristine E 174 samples were purchased online from several producers and suppliers from European countries (Table 3: labelled as Ag-002 to Ag-011). Ag-002, which is a mixture of three food additives, was considered as pristine sample since it did not contain any other matrix components such as sugar or chocolate. Ten food products containing E 174, were bought in Belgian local stores (Table 3: labelled as Ag-P-001 to Ag-P-010). They included a variety of confectionery such as silver-coated sugar beans and silver pearls. Some of the products shown in Table 3, originally bought as silver E 174 products, contain also E 171.

E 171 (titanium dioxide) is a food additive authorized to be used as a food colour in the European Union (EU) (Regulation (EC) No 1333/2008). It is a white powder that is insoluble in water and organic solvents. In food, both anatase and rutile titanium dioxide are applied. E 171 is commonly applied in confectionery (including candies, chewing gum, glazing) (Chen et al., 2013; Faust et al., 2016; Lomer et al., 2000; Peters et al., 2014; Weir et al., 2012), but was found also in pastries, low fat dairy products and sauces (Faust et al., 2016; Lomer et al., 2000; Peters et al., 2014; Weir et al., 2012). Weir et al. (Weir et al., 2012) and Chen et al. (Chen et al., 2013) initially showed that E 171 is a particulate material containing a fraction of nanoparticles.
RTMs NM-100 (anatase, particles in the size range of 100 nm) and NM-103 (rutile, particles in the size range of 20 nm), obtained from the JRC's NM repository as dry powders in glass vials sealed under argon atmosphere, were used to estimate the measurement uncertainties of the median of the minimum Feret diameter distribution of the E 171 samples (Verleysen et al., 2019).
Nine pristine E 171 samples were purchased from web shops specialized in bakery and confectionery products from several countries within the European Union (Table 4: labelled as E 171-01 to E171-09).

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4 Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008, p. 16–33.

5 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.

According to the list of ingredients on the labels, these samples only contained E 171. Six pristine E 171 samples were obtained from business operators (Table 4: labelled as E 171-A to E171-F) and correspond to the same types of E171 for which data were previously evaluated in EFSA (EFSA FAF Panel, 2019). Products containing E 171 (Table 4: labelled as E171-P01 to E171-P25) were bought in European supermarkets, and included several types of confectionery, chewing gum, sugar paste, cake decorations, icing, powdered drinks and sprays. In addition to the products indicated in Table 4, more than 100 drinks and dairy products were screened semi-quantitatively by ICP-MS for the presence of titanium, as a proxy for TiO_2. The drinks were bought in a local supermarket and included milk drinks (from animal and vegetal origin), chocolate drinks, fruit- and vegetable juices, coffee drinks, beers, lemonades and energy drinks, smoothies, coconut drinks and custards with or without chocolate. Additionally, a chocolate sample (80% cocoa) was analysed as well.

Table 2: Overview of analyzed pristine E 175 samples and E 175 containing products.

Reference	Description	Ingredients (translated)
Au-001	Gold flakes	E 175
Au-002	Gold leaves	E 175
Au-P-001	liqueur	E 175, alcohol, Cinnamon flavoring, 331, E418, E327
Au-P-002	liqueur	E 175, water, alcohol, sugar, natural cinnamon flavoring, molasses
Au-P-003	Jam	E 175, Wine of Sauvignon, Sobieski liquor, sugar, natural gelling agent of plants
Au-P-004	Syrup	E 175, cane sugar, water, natural aroma of rose, 0.7% infusion (water, rose flakes 0.3%), citric acid, anthocyanin colorant, possible traces of nuts, gluten, mustard, sesame milk, egg, sulfites and soy

Table 3: Overview of analyzed pristine E 174 samples and E 174 containing products

Reference	Description	Ingredients (translated)
NM-300K	nano-silver RTM	Silver
Ag-001	Powder	Silver
Ag-002	Powder	E 174, E202, E414
Ag-003	Powder	E 174
Ag-004	Flakes	E 174
Ag-005	Flakes	E 174
Ag-006	Petals	E 174
Ag-007	Petals	E 174
Ag-008	Leaves	E 174
Ag-009	Leaves	E 174
Ag-010	Flakes	E 174
Ag-011	Flakes	E 174
Ag-P-001	Sugar beans	Sugar, chocolate (sugar, cocoa mass, cocoa butter, emulsifier: soy lecithin, vanillin), wheat / rice starch, stabilizers: E414-E471, gelatin, flavor, glucose syrup, hardened coconut oil, glazing agents: E901i-E904, anti-caking agents: E553b-E555, thickeners : E460-E464-E472a, preservative: E202, dyes: (E 100, E 122, E 124, E 171, E 174)*
Ag-P-002	Sugar pearls	Sugar, wheat / corn starch, glucose syrup, stabilizer: E414, gelatin, wax-free shellac, (E 100, E 122, E 124, E 171, E 174)*
Ag-P-003	Sugar beans	Sugar, chocolate (sugar, cocoa mass, cocoa butter, emulsifier: soy, lecithin, vanillin), wheat / rice starch, stabilizer: E414, gelatin, flavor, wax-free shellac, (E 100, E 122, E 124, E 171, E 174)*
Ag-P- 004	Sugar beans	Sugar, chocolate (sugar, cocoa mass, cocoa butter, whole milk powder, whey (milk), emulsifier: soy lecithin, vanillin), wheat / rice starch, stabilizer: E414, gelatin, aroma, wax-free shellac, (E 100, E 122, E 124, E 171, E 174)*
Ag-P-005	Sugar beans	Sugar, chocolate (sugar, cocoa mass, cocoa butter, emulsifier: soy, lecithin, vanillin), wheat / rice starch, stabilizer: E414, gelatin, flavor, wax-free shellac, (E 100, E 122, E 124, E 171, E 174)*
Ag-P-006	Sugar beans	Sugar, wheat / corn starch, glucose syrup, stabilizer: E414, gelatin, wax-free shellac, (E 100, E 122, E 124, E 171, E 174)*
Ag-P-007	Sugar beans	Sugar, chocolate (sugar, cocoa mass, cocoa butter, emulsifier: soy lecithin, vanillin), wheat / rice starch, stabilizer: E414, gelatin, flavor, wax-free shellac, (E 100, E 122, E 124, E 171, E 174)*
Ag-P-008	Sugar pearls	Sugar, wheat flour, rice starch, maltodextrin, glazing agent, gum arabic, gelatin (from pig), coloring: silver
Ag-P-009	Sugar pearls	Sugar, rice flour, glucose syrup, coloring: silver, gelatin, salt
Ag-P-010	Sugar beans	Cocoa mass, cocoa butter, sugar, rice starch, maltrodextrin, soy lecithin, gum arabic, carnauba wax, colors: E 174, E 100

*it is stated that depending on the colour of the product, it may contain one or several of these food additives

Table 4: Overview of analyzed pristine E 171 samples and E 171 containing products

Reference	Description	Ingredients (translated)
NM-100	RTM NM-100	TiO_2
NM-103	RTM NM-103	TiO_2
E 171-01	Pristine E171 powder	E 171
E 171-02	Pristine E171 powder	E 171
E 171-03	Pristine E171 powder	E 171
E 171-04	Pristine E171 powder	E 171
E 171-05	Pristine E171 powder	E 171
E 171-06	Pristine E171 powder	E 171
E 171-07	Pristine E171 powder	E 171
E 171-08	Pristine E171 powder	E 171
E 171-09	Pristine E171 powder	E 171
E 171-A	Pristine E171 powder	E 171
E 171-B	Pristine E171 powder	E 171
E 171-C	Pristine E171 powder	E 171
E 171-D	Pristine E171 powder	E 171
E 171-E	Pristine E171 powder	E 171
E 171-F	Pristine E171 powder	E 171
E 171-P01	Button shaped candies	Sugar, cocoa paste, skimmed milk powder, cocoa butter, lactose, milk fats, palm fat, glucose syrup, starch, shea butter, dyes (E 100, E 120, E 132, E 133, E 150a, E 150c, E 150d, E 153, E 160a, E 160e, E 162, E 163, E 171, E 172), dextrins, coating agents (beeswax, carnauba wax), emulsifiers (soy lecithin, E445), coconut oil, salt, aromas
E 171-P02	Chewing gum	Sweeteners (xylitol, isomalt, sorbitol, maltitol syrup, acesulfame-K, sucralose), gum, flavors, stabilizer (E414), emulsifier (sunflower lecithin), colorants (E 171, E 133).
E 171-P03	Marzipan	Sugar glucose, sweetener: E420, invert sugar, almonds (20%), thickener: E466, preservative 202, color: E 171
E 171-P04	Sugar paste	Sugar, glucose syrup, vegetable fat (palm and palm kernels), water stabilizers: glycerol, xanthan gum, E466, emulsifier: E472c, flavor, color: E 171, conversion agent: potassium sorbate. May contain almonds.
E 171-P05	Mints	Sweeteners (sorbitol, xylitol (23%), mannitol, maltitol syrup, maltitol, aspartame, acesulfame-K), gum, filler (E 170), flavorings, humectant (E422), thickener (E414), emulsifiers (E472a, sunflower lecithin) , color (E 171), glazing agent (E903), antioxidant (E321). Contains a source of phenylalanine
E 171-P06	Cake decoration markers	Sugar, water, vegetable oil (palm, rapeseed), coloring foodstuff (spirulina and safflower concentrate), glucose syrup, modified starch, coloring agents (E 171, carmine, lutein), emulsifier (E435), preservative (potassium sorbate), natural flavor, thickener (pectin), acidity regulator (citric acid)
E 171-P07	Confectionery, coated licorice	sugar, molasses, cheat flour, glucose syrop, fully hydrogenated coconut fat, liquorice extract, thickener (gum arabic), humectants (glycerol, sorbitol syrup), ammonium chloride, gelatine, glucose-fuctose syrup, colourant (sulphite ammonia caramel, titanium dioxide), flavo, salt acidity regulator
E 171-P08	Cake decoration	Pearls (16g): Dextrose, sugar, rice flour, glucose syrup, color (E 171), salt, glazing agent (E904), flavor, acid (citric acid), coloring foods (apple, blackcurrant and radish concentrates, vegetable oil (coconut)
E 171-P09	Icing, frosting, glaze	Sugar, glucose syrup, water, preservative (potassium sorbate), color (E 171)
E 171-P10	Coated Biscuit	Sugar, cocoa butter, whole milk powder, wheat flour, rice flour, natural vanilla flavor, emulsifier: soy lecithin, wheat flour, salt, baking agent (E450i, E500ii, wheat flour), glazing agent E414
E 171-P11	Cake decoration	Jade 21g: glucose, cornstarch, dextrin, coating agent (E904, E903), anti-caking agent (E555), food colors (E 100, E 133, E 171).
E 171-P12	Cake decoration	Marble 21g: glucose, cornstarch, dextrin, coating agent (E904, E903), anti-caking agent (E555), food coloring (E 171).
E 171-P13	Icing, frosting, glaze	sugar, partially hydrogenated vegetable fat, water, glucose syrup, wheat starch, emulsifiers (E471, E435), salt, acidity regulators (E330, E575), preservative (E202), flavorings, colors (E 171, E 129, E 122, E 102).
E 171-P14	Confectionery, sugar pearls	dextrose, sugar, glucose syrup, rice flour, glazing agents: E903 and E904, vegetable oil (coconut), salt, natural raspberry flavor, acidity regulator: E330, colorants: E 100, E 153 and E 171, thickener: E414, coloring foods: radish (a), lemon (b), black currant (c)
E 171-P15	Chewing gum	Sweeteners xylitol, sorbitol, aspartame, mannitol, acesulfame-K; gum base, gum arabic thickener, flavors, humectant glycerol, soy lecithin emulsifier, coloring E 171, coating agent carnauba wax, antioxidant BHA. Contains a source of phenylalanine
E 171-P16	Instant powder orange drink	sugar, acidifier (citric acid), coloring (E 100, E 171), salt, acidity regulator (E331), sweeteners (asparthame, acesulfame K), thickener (E466), vitamin C, natural orange flavor (contains lecithins of soy), vitamin A, concentrated black carrot juice, folic acid, vitamin B2. Contains a source of phenylalanine
E 171-P17	Mints	sweeteners (isomalt, sorbitol, xylitol, steviol glycosides), flavor, color (E 171), natural flavorings (mint), acidifier (citric acid), acidity regulators (citric acid, potassium tartrate), glazing agent (carnauba wax)
E 171-P18	Chocolate confectionery with peanuts	Peanuts (24%) in milk chocolate (48%) in a colored sugar coating, sugar, peanuts, cocoa mass, skimmed milk powder, palm fat, cocoa butter, lactose and milk proteins, butterfat (from milk), shea fat, salt, coloring (E 100, E 120, E 133 , E 160a, E 160e, E 171), glucose syrup, starch, emulsifier (soy lecithin), dextrin, glazing agent (carnauba wax), flavorings. May Contain: Hazelnut, Almond)
E 171-P19	Chocolate confectionery with puffed rice	Puffed rice (17%) in milk chocolate (62%) in a colored sugar coating, sugar, cocoa mass, rice flour (11%), skimmed milk powder, palm fat, cocoa butter, lactose, butterfat (from milk), shea fat, salt, barley malt extract, dyes (E 100, E 120, E 133, E 160a, E 160e, E 171), glucose syrup, starch, emulsifier (soy lecithin), dextrin, dextrose, glazing agent (carnauba wax), flavorings, coconut oil. May Contain: Peanut, Hazelnut, Almond)
E 171-P20	Croissant filling	soft wheat flour, fresh milk cream 25% [glucose syrup, sugar, water, pasteurized fresh milk (10% in the cream, corresponding to 2.5% of the total), palm vegetable fat, skimmed milk in powder, flavorings, thickeners : pectin, colors: E 171, preservatives: potassium sorbate] vegetable margarine (palm fat, water, sunflower oil), sugar, fresh eggs (4.8%), natural yeast (gluten), cereal flour (2, 5%) (oats, rice, barley, spelled, buckwheat), emulsifiers: mono-and diglycerides of fatty acids, skimmed milk powder, salt, flavorings, malted barley flour. It may contain traces of nuts and soy
E 171-P21	Toothpaste	Aqua, sorbitol, hydrated silica, glycerin, potassium Nitrate, PEG-6, Cocamidopropyl Betaine, Aroma, Titanium Dioxide, xanthan gum, Sodium Saccharin, Sodium Fluoride, Sodium Hydroxide, Limonene
E 171-P22	Cake decoration spray Baby blue	Propellants (Butane E943a, Isobutane E943b, Propane E944) Ethyl Alcohol, Colourings: E 171, E 133. Aluminum contained in 188 g of sprayed product: 150mg.
E 171-P23	Cake decoration spray Silver	Propellants (Butane E943a, Isobutane E943b, Propane E944), Ethyl Alcohol, Colourings: E 171, E 172.
E 171-P24	Cake decoration spray Pearl	Propellants (Butane E943a, Isobutane E943b, Propane E944), Ethyl Alcohol, Flavours, Colourings: E 171.
E 171-P25	Cake decoration spray Pink	Propellants (Butane E943a, Isobutane E943b, Propane E944), Ethyl Alcohol, Flavours, Colourings: E 171, E 129.

2.2. Methodologies

Methods are developed, validated and applied for the characterization of the fraction of nanoparticles in the food additives E 171, E 174 and E 175. The characterization methodology combines physical characterization by electron microscopy-based techniques, such as TEM, SEM and HAADF-STEM, with chemical characterization by spectroscopic techniques, such as EDX, ICP-MS and spICP-MS. Typically the following steps are followed in a complete analysis (Figure 2):

- Sample preparation and screening for the presence of nanoparticles in food additives and food products by descriptive EM analysis and ICP-techniques (WP1).
- Confirmation of the chemical identity by electron diffraction, EDX and spICP-MS (WP2).
- Measurements of the number based size and shape distributions of the constituent particles in the food additives by EM (WP3).
- Determination of the concentration of the fraction of nanoparticles by ICP-MS/ICP-OES and spICP-MS (WP4).

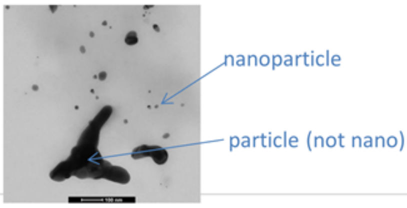
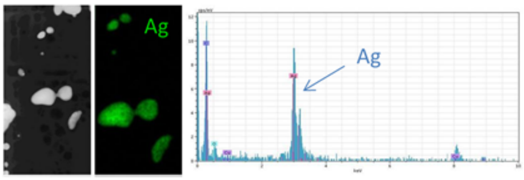
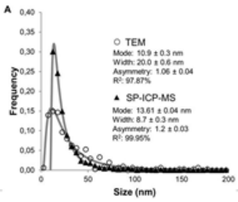
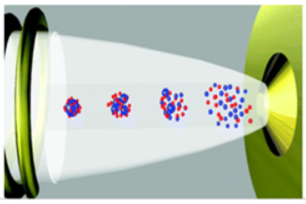
<table>
<tr><th>Sub-question</th><th>Methodology</th><th>Illustration</th></tr>
<tr><td>WP1: Are particles present in the food additives and products</td><td>Descriptive TEM</td><td>
</td></tr>
<tr><td>WP2: What is the chemical composition of the individual particles in food additives and products?</td><td>HAADF-STEM + EDX</td><td>
</td></tr>
<tr><td>WP3: Are the particles of the food additives and products nano?</td><td>Quantitative TEM</td><td>
</td></tr>
<tr><td>WP4: What is the concentration of the (nano)particles in the food additives and products?</td><td>Total mass concentration: ICP-MS
Mass and number concentration of particles: SP-ICP-MS</td><td></td></tr>
</table>

Figure 2 Illustration of the steps in a complete analysis of the fraction of nanoparticles in a food additive or food product.

2.2.1. Sample preparation

The sample preparation of the food additives in their pristine state and in food products is described in detail by (De Vos et al., 2020; Verleysen et al., in preparation, 2020; Waegeneers et al., 2019). Dispersion protocols including pH optimization based on zeta potential, probe sonication and centrifugation were described in detail in SOPs.

2.2.2. ICP-OES and ICP-MS

ICP-MS or ICP-OES was applied to determine the total concentrations of silver (Ag), titanium (Ti) or gold (Au) in the food additive containing products. Samples were measured using the Agilent 8800 triple quadrupole ICP-MS (Agilent, Belgium), equipped with a non-quartz sample introduction system consisting of a perfluoroalkyl (PFA) Microflow nebulizer, a 2.5 mm ID sapphire injector and a PFA spray chamber.

2.2.3. TEM specimen preparation

TEM specimens were prepared using Alcian blue treated positively charged pioloform- and carbon-coated, 400 mesh copper grids (Agar Scientific, Stansted, Essex, UK), by drop deposition (NANoREG, 2013).

2.2.4. Descriptive TEM analysis

A set of calibrated images that representatively show the distribution of the material on the TEM specimen was recorded by a 120kV Tecnai G2 Spirit TEM with BioTwin lens configuration (Thermo Fisher Scientific, Eindhoven, The Netherlands), equipped with a 4 × 4 k Eagle charge-coupled device (CCD) camera (Thermo Fisher Scientific, Eindhoven, the Netherlands) while using the TEM imaging and analysis (TIA) software (Version 3.2, Thermo Fisher Scientific, Eindhoven, The Netherlands). All samples were initially screened at multiple magnifications and a detailed description of all examined TEM specimens was prepared based on these images, allowing to assess the quality of the specimen preparation as described by (Barrett, 1980; Juan Lopez-de-Uralde et al., 2010; Munoz-Marmol et al., 2015).

2.2.5. Elemental analysis

SEM imaging and EDX analyses were performed using a JEOL JSM–7800F 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 AZTec® EDS software NanoAnalysis (version, Oxford Instruments, High Wycombe, UK) was used.

HAADF-STEM imaging and EDX analyses were perfomed using a 200kV Talos F200S G2 TEM equipped with an HAADF detector and Super-X EDS detector consisting of 2 windowless silicon drift detectors (SDD), using Velox software (ThermoFisher, Eindhoven, The Netherlands).

2.2.6. Crystallographic structure

The crystallographic structure of the materials was determined by electron diffraction using both the Tecnai and Talos microscopes. Diffraction patterns of regions containing many particles were recorded, indexed and compared to a database (Yibin et al., 2011). The camera length was determined based on colloidal gold nanoparticles.

2.2.7. Quantitative TEM: imaging and image analysis

Quantitative TEM analysis was performed using the Tecnai microscope and aims to record a set of calibrated transmission electron images that representatively show the material on the TEM specimen (NANoREG, 2013). The SOP foresees that the images are randomly and systematically recorded, at 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. To determine unbiased, number-based size distributions by quantitative TEM for all samples, the magnification and the associated pixel size were determined based on the criterion of Merkus (Merkus, 2009) determining the lower limit of quantification (LLOQ). The corresponding upper limit of quantification (ULOQ) is limited to one tenth of the image size supporting on ISO 13322-1 (Particle Size Analysis - Image Analysis Methods - Part 1: Static Image

Analysis Methods, 2004). The size and shape properties of the constituent particles of the materials were measured based on the properties of their 2D projections using the 'NanoDefine ParticleSizer' software (Mech et al., 2020). The constituent particles were analysed by noise and background suppression combined with irregular watershed mode or ellipse fitting mode. Particles that were clearly not correctly measured were removed manually from the datasets.

The raw data resulting from the image analysis was processed using an in-house python script for calculation of descriptive statistics and making histograms, following ISO 9276-1 guidelines for representation of results of particle size analysis (Representation of Results of Particle Size Analysis - Part 1: Graphical Representation, 1998). For all materials, the number-based distributions of the minimum Feret Diameter, Feret diameter, and the aspect ratio of the constituent particles were determined. Bin width was calculated using Freedman-Diaconis rule.

Instead of a parametric approach suggested by ISO 9276-3 (Representation of Results of Particle Size Analysis - Part 3: Adjustment of an Experimental Curve to a Reference Model, 2008), a non-parametric approach was applied. The normalized number-based distributions and the kernel density estimates (KDE) of the Fmin, Fmax, and AR parameters were determined using the seaborn.distplot function. For the KDE plot, producing a continuous density estimate, a Gaussian kernel was applied. The modes of the distributions were calculated from the KDE. For each sample, a statistics file including the median, mean and modal values of the distributions was generated.

For pristine E171 samples and products containing E171, the percentage of particles with a minimum Feret diameter smaller than a specific size in the range of 10 nm to 500 nm was determined from the image analysis datasets for each sample, using an in-house python script. In addition, the corresponding mass percentages of these fractions were calculated. The short (a-axis) and long axes (c-axis) of the ellipse fitted to the 2D projection of each constituent particle was measured as a proxy of its minimum and maximum external dimension, respectively. Assuming that the particles are prolate ellipsoids (a-axis = b-axis < c-axis), the volume (V) of each particle was estimated as (eq. 1):

$$V = \frac{4}{3}\pi a^2 c \qquad \text{(eq. 1)}$$

and the mass (M) of each particle was calculated as (eq. 2):

$$M = V\rho \qquad \text{(eq. 2)}$$

with ρ = the density of the particles (The calculated bulk density of anatase TiO_2 = 3.89 g/cm^3, and of rutile TiO_2 = 4.23 g/cm^3).

2.2.8. spICP-MS

spICP-MS was applied to determine the size, number concentration and mass concentration of the particles of interest present in the pristine samples and in the food additive containing products. An Agilent 8800 ICP-MS/MS (Agilent Technologies) was used for data acquisition in time-resolved analysis mode.

2.2.9. TEM measurement uncertainties

For E 171, the uncertainties associated with the quantitative TEM analyses were determined in the Nanodefine and joint Nanofood@-EFSAnano projects and contained the within day variability (repeatability), the between days variability, the calibration uncertainty and the trueness uncertainty. The validation parameters were stability, working range, selectivity, ruggedness, precision, measurement uncertainty and the limit of quantification. For TiO_2 representative test materials NM-100 and NM-103, the expanded measurement uncertainty on the median of the minimum Feret diameter distribution was reported to be 8.5% and 9.2%, respectively (Verleysen et al., 2019). For pristine E 171 samples, similar measurement uncertainties were expected. These validation studies were reported in detail by Verleysen et al. (Verleysen et al., 2019, 2020)

For E 174, stability, precision and measurement uncertainty were determined within the joint Nanofood@-EFSAnano project. In the validation study of the representative test material NM-300K, the measurement of four physical parameters was evaluated: the minimum Feret diameter as a measure for the minimal external dimension, the equivalent circular diameter (ECD) as a size parameter that allows comparing measurement results with other techniques, the aspect ratio as a shape parameter and the solidity as a surface topology parameter. To estimate the measurement uncertainty on these parameters for E 174, the uncertainty of the sample preparation was taken into account. This was reported in detail by De Vos et al. (De Vos et al., 2020)

2.2.10. spICP-MS and ICP-MS/OES measurement uncertainties

To determine the uncertainties associated with the analysis of TiO_2 nanoparticles by spICP-MS, validation studies have been performed on the materials NM-100 and NM-103, and on Ag-P-004 (this is a E 174 and E 171 containing product, and was applied to determine measurement uncertainties for both food additves). Results are reported in detail by Verleysen et al. (Verleysen et al., 2020)

To determine the uncertainties associated with the analysis of Au nanoparticles by spICP-MS, validation studies have been performed on NIST RM 8012 (Kaiser & Watters, 2007b).

To determine the uncertainties associated with the analysis of Ag nanoparticles by spICP-MS, validation studies have been performed on the material NM-300K and on Ag-005 and Ag-009 (pristine materials), Ag-P-003 and Ag-P-004 (E 174 containing products) and a partial revalidation of NM-300K due to a slightly adapted analytical methodology. This is reported in detail by Waegeneers et al. (Waegeneers et al., 2019).

Setting up a complete uncertainty balance is hampered by the lack of certified values of reference materials for particles in food additives: the uncertainty associated with trueness cannot be assessed and can only be estimated based on other certified reference materials. No certified reference materials (only RTMs) are available for silver and titanium dioxide particles, while the size and uncertainty values reported for the Au nanoparticles from NIST RM 8011 (Kaiser & Watters, 2007a), RM 8012 (Kaiser & Watters, 2007b), RM 8013 (Kaiser & Watters, 2007c) are only indicative.

Uncertainties associated with the analysis of total Ag by ICP-OES, and of total Au and Ti by ICP-MS were determined according to the internal ISO 1725 compliant quality system of Sciensano.

3. Results

3.1. Physicochemical characterization of the E 174 food additive

The results of the analyses performed on E 174 are summarized in the publications of Waegeneers et al. (Waegeneers et al., 2019) and De Vos et al. (De Vos et al., 2020).

3.1.1. Methodology development and validation

Waegeneers et al. (Waegeneers et al., 2019) focused on the methodology development and validation of the method. For TEM, a validation study of the representative test material NM-300K was performed and the measurement of four physical parameters was evaluated: the minimum Feret diameter as a measure for the minimum external dimension, the ECD as a size parameter that allows comparing measurement results with other techniques, the aspect ratio as a shape parameter and the solidity as a surface topology parameter (Waegeneers et al., 2019). The respective expanded measurement uncertainties were 12%, 11%, 7.0% and 6.7%. To estimate the measurement uncertainty on these parameters for E 174, the uncertainty of the sample preparation was taken into account. This uncertainty was estimated to be 6.8%, resulting in a combined expanded uncertainty of 18% on the minimum Feret diameter determined in E 174 (De Vos et al., 2020).

For spICP-MS, the expanded measurement uncertainty for Ag nanoparticle sizing was calculated to be 16% in E 174-containing food products and increased up to 23% in pristine E 174. The E 174 samples showed a large silver background concentration combined with a low number of nanoparticles, making data interpretation more challenging than in the E174 containing products. The standard uncertainties related to sample preparation, analysis, and challenging data interpretation were respectively 4.7%, 6.5%, and 6.0% for triplicate performances. For a single replicate sample, the uncertainty related to sample preparation increased to 6.8%. The expanded measurement uncertainty related to the concentration determination was 25–45% in these complex samples, without a clear distinction between pristine samples and E174-containing products. Overall, the validation parameters obtained for spICP-MS seem to be fit for the purpose of characterizing silver nanoparticles in E 174 or E 174-containing products."

3.1.2. Physicochemical characterization of the fraction of nanoparticles in pristine E 174 and E 174 containing products.

The publication of De Vos et al. (De Vos et al., 2020) focused on the application of the methods described by Waegeneers et al. (Waegeneers et al., 2019) to characterize the fraction of nanoparticles in pristine E 174 samples and E 174 containing products:

The study characterized the fraction of silver nanoparticles in 10 commercially available pristine E 174 samples and 10 E 174-containing products by TEM and spICP-MS. TEM analysis showed that all samples (10 E174 samples and 10 products containing E174) contained micrometer-sized flakes and also a fraction of nanoparticles. Energy-dispersive X-ray spectroscopy (EDX) and electron diffraction confirmed that the nanoparticles and micrometer-sized flakes consisted of silver. A higher amount of particles was observed in the E 174-containing products than in the pristine E174 samples. In addition, the surface of the micrometer-sized flakes was rougher in products. The median of the minimum external dimension, assessed as minimum Feret diameter, of the fraction of particles determined by quantitative TEM analysis was 11 ± 4 nm and 18 ± 7 nm (overall mean ± standard deviation), for pristine E174 samples and E174-containing products, respectively. In general, 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 particles determined by spICP-MS was 19 ± 4 nm and 21 ± 2 nm (overall mean ± standard deviation), for pristine E174 samples and E174-containing 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."

3.2. Physicochemical characterization of the E 175 food additive

The methodology for the screening, detection and physicochemical characterization of nanoparticles in E 174 was applied for the screening of pristine E 175 samples and food products containing E 175. Because gold is very stable, problems associated with instability, as observed for E 174, did not occur. ICP-MS of Au-P-001 and Au-P-002 demonstrated that the element gold was present in these products (total gold concentrations were 30 ± 6 mg/L and 23 ± 5 mg/L, respectively). No nano-sized gold particles were detected in pristine E 175 samples and food products containing E 175 by TEM and spICP-MS.

3.3. Physicochemical characterization of the E 171 food additive

3.3.1. Methodology development and validation

To determine the uncertainties associated with the analysis of TiO_2 nanoparticles by spICP-MS, validation studies have been performed on RTMs NM-100 and NM-103, and on Ag-P-004 (E 174 and E 171 containing products). Precision parameters were determined by analyzing three independent replicates of RTM NM-100 (JRC, ISPRA, Italy) on each of five different days, and assessed via one-way analysis of variance (Verleysen et al., 2020). The measurement uncertainty budgets of particle sizing by spICP-MS was in the order of 16 % (Ucx, k=2).

A general approach for the size measurement of particulate (nano)materials by transmission electron microscopy was evaluated by (Verleysen et al., 2019). The methodologies and their validation reports with measurement uncertainties are applicable also for titanium dioxide, because RTMs NM-100 (anatase TiO_2 with particles in the 100 nm size range) and NM-103 (rutile TiO_2 with particles in the 20 nm size range) were included in the study. Expanded measurement uncertainties (k=2) of 8.5% and 9.2% were reported for NM-100 and NM-103, respectively. Based on these validation studies, the measurement uncertainty budget of particle sizing of E 171 by TEM is expected to be in the order of 10% (Ucx, k=2).

3.3.2. Physicochemical characterization of the fraction of nanoparticles in pristine E 171

The results of the physicochemical analyses performed on pristine E 171 are summarized in the publication of Verleysen et al. (Verleysen et al., 2020) who applied standardized and validated methodologies to characterize representative samples of 15 pristine E 171 materials based on TEM and spICP-MS.
To measure the minimum external dimension of the constituent particles of E 171, sample preparation factors influencing particle dispersion (pH, medium, probe sonication and centrifugation) were tested and optimized on 15 pristine E 171 samples (Table 4), including 9 E171 samples available on the public market (E171-01 – 09) and 6 E 171 samples obtained from business operators (E171-A – F) (Verleysen et al., 2020). The evaluation of selected sample preparation protocols allowed identifying and optimizing the critical factors that determine the measurement of the particle size distribution by TEM.

Electron diffraction demonstrated that both anatase and rutile TiO_2 particles are sold as E 171, including 3 samples containing smaller rutile TiO_2 particles (20-40 nm) coated with mica (potassium aluminium silicate) (Table A.1). 'Potassium aluminium silicate-based pearlescent pigments' are mentioned in the EFSA opinion on the re-evaluation of E555 where it is indicated "Interested business operators stated that 'potassium aluminium silicate-based pearlescent pigments' (e.g. potassium aluminium silicate coated with titanium dioxide) used on a food product is labelled as E 171 or titanium dioxide" and the EFSA FAF Panel concluded that "potassium aluminium silicate in 'potassium aluminium silicate-based pearlescent pigments' does not meet the definition of a carrier according to Regulation (EC) 1333/2008 and 'potassium aluminium silicate-based pearlescent pigments' are not listed in Regulation (EC) 1333/2008. The Panel also concluded that 'potassium aluminium silicate-based pearlescent pigments' should be evaluated as a new food additive" (EFSA FAF Panel (Panel on Food Additives and Flavourings), 2020), which are potassium aluminium silicate-based pearlescent pigments (EFSA FAF Panel (Panel on Food Additives and Flavourings), 2020).

In optimized conditions, all E 171 samples were shown to contain an important fraction of nanoparticles. The large majority of constituent particles, confirmed to be TiO_2 by EDX, were reliably detected and measured using the applied ParticleSizer software. In the most dispersed state, the particle size distributions obtained by TEM and spICP-MS agreed well. Using the optimized sample preparation protocols and image analysis settings, 12 of the 15 samples showed a median minimum external dimension below 100 nm. When the expanded measurement uncertainties (U_{cx}, k = 2) were considered, 11 samples had a median minimum external dimension significantly smaller than 100 nm (median $F_{min} + U_{cx}$ (k = 2) < 100 nm). TiO_2 particle concentrations estimated by spICP-MS were in the range of 10^{17} particles per kg TiO_2.
Appendix A.1 shows that for the 15 pristine materials, the percentage of nanoparticles (minimum Feret diameter smaller than 100 nm) ranged from 18% to 74% (pearlescent pigments excluded). The corresponding mass percentages of these fractions ranged from 2% to 32%. Hardly any particles smaller than 30 nm were observed (pearlescent pigments excluded).

3.3.3. E 171 homogeneity study

A pristine E 171 intra-laboratory reference material was produced within the EFSAnano project. 300 homogeneous and stable vials of the pristine E 171 intra-laboratory reference material were fractionated (collaboration with JRC Ispra). Sub-fractions of the same batch are available to produce more vials when necessary.

The report of the homogeneity study "Characterization of an Intra-laboratory reference material by transmission electron microscopy: pristine E 171 (JRCNM14500a)" was published as supplementary information in the publication of Geiss et al. (Geiss et al., 2021), where this E 171 reference material was used in an interlaboratory comparison study for spICP-MS among seven experienced European food control and food research laboratories, organized by JRC.

3.3.4. Physicochemical characterization of the fraction of nanoparticles in E171 containing products.

The methodology developed to characterize pristine E 171 food additives can be applied to characterize the particles isolated from products containing E 171, provided that EM grids contain a sufficient number of well-dispersed (no or little agglomeration) particles that are homogeneously distributed on the grid and that are representative for the material. This generally requires optimization of the sample preparation by bringing the particles in a dispersion that is suitable to prepare a representative EM specimen fit for quantitative EM analysis.

In the project, such a sample preparation methodology was developed to characterize the nanosized TiO_2 particles in E 171 containing products. For relatively simple food matrices, such as confectionery with a sugar coating containing E 171, adaptation of pH, sonication energy and centrifugation speed and time allowed obtaining representative samples of TiO_2 particles. For more complex matrices, partial removal of the matrix using KOH, combined with optimized pH, sonication energy and centrifugation speed and time was required to release particles from the matrix and ensure representative sampling.

A generic protocol for sample preparation and physicochemical characterization of food additive E 171 in a food matrix was developed (Verleysen et al., in preparation). The protocol was applied on 20 different types of confectionery and 1 personal care product, available on the European market (Table 4). The protocol combines a generic sample preparation protocol for matrix extraction and representative sampling with total concentration measurement by ICP-MS and particle size, shape and elemental composition measurement by electron microscopy based techniques. The protocol allowed representative sampling for 20 food products (E171-P01 - P20) and 1 personal care product (E171-P21). For 1 food product (E171-P13), the concentration measured by ICP-MS was too low to allow representative sampling by TEM without adjusting the protocol. In 5 food products (E171-P08, P10, P11, P12 and P14), labelled to contain E 171, only pearlescent pigments were observed. For the remaining 15 products, including 14 food products (E171-P01-P07, P09, P15-P20), and the personal care product (E171-P21), homogeneous, well concentrated and representative distributions of particles on the grids were obtained. The methodology can be easily extended for analysing TiO_2 in cosmetics, personal care products and medicines. Appendix A.2 shows that for the 15 analyzed products, the percentage of nanoparticles (minimum Feret diameter smaller than 100 nm) ranged from 23% to 82%. The corresponding mass percentages of these fractions ranged from 3% to 41%. Hardly any particles smaller than 30 nm were observed.

In addition, edible sprays labelled to contain food additives including E 171, E 172 and E555 (Table 4: E171-P22 – E171-P25) were examined by TEM and EDX. The presence of all poorly soluble, biopersistent, particulate compounds was assessed. It was shown that the sprays all contained pearlescent pigments. The results of this study are submitted for publication (Mathioudaki et al., submitted).

Since E 171 is patented to whiten drinks, in addition to the samples that were labelled with E 171, more than 100 drinks and dairy products were analysed semi-quantitatively for the presence of titanium, as a proxy for TiO_2 (Table 5). The drinks were bought in a local supermarket and included milk drinks (from animal and vegetal origin), chocolate drinks, fruit- and vegetable juices, coffee drinks, beers, lemonades and energydrinks, smoothies, coconut drinks and custards with or without chocolate. Additionally, a chocolate sample (80% cocoa) was analyzed as well. The Ti concentration in all products was low compared to the Ti concentration in products to which E 171 is added. Products to which cocoa/chocolate is added contain more Ti than other products. This is probably due to the Ti present in cocoa, as the analyzed chocolate sample contained about 7 mg Ti/kg. The Ti concentration in tomato- and vegetable juices was variable (<0.020 – 0.52 mg/kg). The highest concentration was found in a multi-vegetable juice. The Ti concentrations in milk drinks and smoothies were low but detectable. In beer, no Ti could be detected except for one sample (0.31 mg/kg). This may be due to contamination during the production process. Overall, the measured Ti concentrations seem to correspond to natural Ti levels in food products.

Table 5: Semi-quantitative screening for Ti in drinks and dairy products.

Product group	**Ti (mg/kg)***			
	Median	**Mean**	**Max**	**n**
Chocolatdrinks (cow milk, soymilk, drinkyoghurt, ricemilk)	0.40	0.44	0.94	12
Tomato- and vegetable juice	0.057	0.16	0.52	4
Milkdrinks (cow milk, soymilk, drinkyoghurt, ricemilk, almondmilk-, oatmilk)	0.043	0.044	0.087	36
Fruitsmoothies	0.032	0.038	0.078	6
Beers	<0.020	<0.020	0.31	19
Coffeedrinks	<0.020	<0.020	0.059	7
Coconut milk and water	<0.020	<0.020	<0.020	4
Soft drinks, energydrinks, sports drinks	<0.020	<0.020	<0.020	18
Fruitjuices	<0.020	<0.020	<0.020	8
Pudding without chocolate	-	0.055	0.079	2
Pudding with chocolate	-	1.1	-	1

*Detection limit: 0.020 mg/kg

Besides the reported physicochemical characterizations, TEM analyses were performed, as a golden standard, in the context of the inter-laboratory comparison study for spICP-MS on pristine E 171 samples and E 171 containing food products (Geiss et al., 2021). These experiments aimed to characterize the titanium oxide particles from a powdered pristine E 171 material, dispersed by probe sonication (10kJ), and two food matrices containing E 171 (chewing gum and button shaped confectionery).

4. Conclusions

The joint nanofood@ - EFSAnano project developed analytical methodologies that allow identification and characterization of nanoparticles in food additives in their pristine state and in the food matrix. The results demonstrated useful application of these metrological sound, validated methods, in a regulatory context, for control and risk identification purposes.

The proposed approach is useful to implement the particle characterisation required by the "EFSA guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles"[6] in preparation, and the "EFSA guidance on the human and animal risk assessment of the application of nanoscience and nanotechnologies in agri/food/feed." (EFSA Scientific Committee, 2018). In this perspective, it can contribute to the re-evaluation and assessment of authorised food additives.

The project allowed Sciensano to continue to play a role in the capacity building activity aiming at supporting Member State Competent Authorities in their enforcement's duties. In particular, the "Nano in Food" project of Member State official control laboratories, supported by JRC, to test compliance with the legislative requirements related to (engineered) nanomaterials in food or the presence in food of materials in the nanoscale, which do not meet the engineered nanomaterial definition set out in Regulation (EU) 2015/2283 ("Regulation 2015/2283 of the European Parliament and of the Council of 25 November 2015 on Novel Foods, Amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and Repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001," 2015), but which may contain a nanoparticle fraction, was supported.

The methodologies and expertise developed in the project form a strong base to fulfil control activities and to provide expertise in the characterization of nano-sized particles in materials which may contain a fraction of nanoparticles, applied in the food chain. In view of further expertise and capacity building to characterise nanomaterials sustainably and on the long term in Sciensano, the expertise built up in this project forms an important base for continued participation to international activities, such as initiatives taken by EFSA, JRC and DG-SANTE, and for activities as national reference laboratory.

[6] https://www.efsa.europa.eu/en/consultations/call/public-consultation-draft-efsa-guidance-technical-requirements

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Glossary and Abbreviations

Ag:	Silver
Au:	Gold
CCD:	Charge-coupled device
EC:	European Commision
ECD:	Equivalent circular diameter
EDS:	Energy dispersive X-ray spectroscopy
EDX:	Energy dispersive X-ray spectroscopy
EM:	Electron microscopy
ESD:	Mass equivalent spherical diameter
EU:	European Union
Fmax or Feret Max	Maximum Feret diameter, estimation of maximal external dimension
Fmin or Feret Min:	Minimum Feret diameter, estimation of minimal external dimension
FPS:	Federal Public Service
HAADF-STEM:	High angle annular dark field Scanning transmission electron microscopy
ICP-MS:	Inductively coupled plasma - mass spectrometry
ICP-OES:	Inductively coupled plasma – optical emission spectroscopy
KDE:	Kernel density estimation
LLOQ:	lower limit of quantification
MDS:	Material datasheet
NM:	Nanomaterial
PFA:	Perfluoroalkyl
RTM:	Representative test material
SEM:	Scanning EM
SOP:	Standard operating procedure
spICP-MS:	Single particle inductively coupled plasma - mass spectrometry
TIA:	TEM imaging and analysis (software)
TEM:	Transmission EM
Ti:	Titanium
TiO2:	Titanium dioxide
ULOQ:	upper limit of quantification

A.1. Number- and mass-based percentages of particles with a minimum Feret diameter smaller than a specific size in the range of 10 nm to 500 nm in a series of 15 pristine materials labelled as E 171.

Fmin (nm)	% of particles smaller than the indicated Fmin value														
	E 171-01*	E 171-02	E 171-03	E 171-04	E 171-05*	E 171-06	E 171-07	E 171-08*	E 171-09	E 171-A	E 171-B	E 171-C	E 171-D	E 171-E	E 171-F
10	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	19	0	0	0	46	0	0	63	0	0	0	0	0	0	0
30	49	1	0	0	78	1	1	89	1	0	0	0	0	0	0
40	75	6	1	2	91	2	8	96	3	1	2	1	1	2	0
50	87	13	4	5	96	5	16	98	7	3	6	3	1	6	2
60	95	22	9	12	99	13	25	99	15	6	15	9	2	14	3
70	98	36	21	24	99	25	37	99	29	11	29	19	4	24	7
80	100	50	35	39	100	38	51	100	44	20	44	31	8	40	9
90	100	63	50	54	100	52	63	100	58	30	58	44	13	54	14
100	**100**	**74**	**64**	**67**	**100**	**65**	**73**	**100**	**71**	**40**	**70**	**56**	**18**	**65**	**20**
110	100	81	75	77	100	74	80	100	79	50	79	67	24	75	28
120	100	87	83	85	100	82	86	100	85	60	85	75	31	83	35
130	100	92	89	90	100	89	91	100	90	68	90	82	38	88	43
140	100	94	92	94	100	93	94	100	94	77	93	88	44	91	51
150	100	97	95	96	100	96	96	100	96	83	95	92	50	95	59
160	100	98	97	98	100	97	98	100	97	88	97	95	57	97	67
170	100	98	98	99	100	99	98	100	98	91	98	97	65	98	76
180	100	99	99	99	100	99	99	100	99	93	99	98	72	99	81
190	100	99	100	100	100	100	99	100	99	96	99	99	76	99	87
200	100	100	100	100	100	100	100	100	100	97	100	99	83	100	91
250	100	100	100	100	100	100	100	100	100	100	100	100	95	100	99
300	100	100	100	100	100	100	100	100	100	100	100	100	98	100	100
400	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
500	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

*pearlescent pigment

Fmin (nm)	% of mass of particles smaller than the indicated Fmin value														
	E 171-01*	E 171-02	E 171-03	E 171-04	E 171-05*	E 171-06	E 171-07	E 171-08*	E 171-09	E 171-A	E 171-B	E 171-C	E 171-D	E 171-E	E 171-F
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	1	0	0	0	6	0	0	13	0	0	0	0	0	0	0
30	11	0	0	0	22	0	0	31	0	0	0	0	0	0	0
40	33	0	0	0	40	0	0	47	0	0	0	0	0	0	0
50	51	1	0	0	55	0	1	57	1	0	1	0	0	0	0
60	75	3	1	2	70	2	3	67	2	0	2	1	0	2	0
70	89	7	4	5	77	5	7	74	6	1	6	3	0	4	0
80	100	14	9	11	81	10	13	81	12	3	12	7	0	10	1
90	100	23	18	20	85	18	22	85	21	6	20	13	1	18	2
100	**100**	**33**	**29**	**32**	**85**	**29**	**31**	**90**	**32**	**10**	**30**	**20**	**2**	**27**	**3**
110	100	44	41	43	90	39	41	91	43	15	41	30	3	38	6
120	100	54	52	56	93	50	52	91	52	23	50	39	5	49	9
130	100	64	63	66	93	64	61	95	62	30	59	49	8	58	13
140	100	71	70	75	93	72	70	100	73	40	68	59	11	66	19
150	100	80	79	81	93	82	78	100	78	49	73	69	15	75	27
160	100	84	84	88	100	87	84	100	84	58	80	77	20	82	35
170	100	88	90	92	100	91	88	100	88	65	85	84	27	86	47
180	100	92	93	95	100	95	92	100	92	70	90	89	34	90	55
190	100	94	96	96	100	96	95	100	95	77	94	92	40	93	65
200	100	95	98	99	100	98	96	100	97	82	96	95	49	96	74
250	100	100	99	100	100	100	100	100	100	95	100	100	75	99	96
300	100	100	100	100	100	100	100	100	100	98	100	100	88	100	100
400	100	100	100	100	100	100	100	100	100	100	100	100	99	100	100
500	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

*pearlescent pigment

A.2. Number -and mass-based percentages of particles with a minimum Feret diameter smaller than a specific size in the range of 10 nm to 500 nm in a series of 15 products containing E 171.

Fmin (nm)	% of particles smaller than the indicated Fmin value														
	E 171-P01	E 171-P02	E 171-P03	E 171-P04	E 171-P05	E 171-P06	E 171-P07	E 171-P09	E 171-P15	E 171-P16	E 171-P17	E 171-P18	E 171-P19	E 171-P20	E 171-P21
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	1	1	1	1	0	1	0	0	1	0	0	1	1
30	1	1	1	1	1	2	1	1	0	0	3	1	1	3	2
40	2	2	2	3	2	3	3	3	2	1	7	2	2	5	4
50	3	4	4	6	2	5	5	8	3	5	16	7	8	7	7
60	7	8	9	11	3	9	9	19	7	9	29	12	17	14	11
70	12	17	16	19	6	18	14	34	12	17	46	20	32	21	19
80	21	28	26	30	10	30	25	48	19	29	61	32	46	30	28
90	30	41	35	41	16	45	33	60	29	44	73	43	60	38	41
100	**40**	**52**	**45**	**51**	**23**	**59**	**45**	**69**	**38**	**57**	**82**	**53**	**71**	**47**	**50**
110	48	65	54	61	30	69	55	78	48	64	88	63	79	53	60
120	58	75	63	68	37	78	64	84	59	72	92	71	86	60	69
130	66	81	71	76	45	82	71	87	66	78	94	79	91	68	77
140	73	87	78	82	53	87	79	91	74	81	96	84	94	75	82
150	79	90	83	85	60	89	84	94	81	85	97	87	96	80	87
160	85	93	89	88	65	93	88	95	86	89	98	90	97	84	90
170	88	95	91	90	71	95	90	96	89	93	99	92	98	89	93
180	91	96	94	93	77	96	93	97	91	95	99	94	99	91	95
190	94	97	95	95	83	97	95	98	93	97	100	96	99	92	97
200	95	98	96	96	86	99	97	99	95	98	100	98	100	94	98
250	99	100	99	99	96	100	99	100	99	100	100	100	100	99	100
300	100	100	100	100	99	100	100	100	100	100	100	100	100	100	100
400	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
500	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Fmin (nm)	% of mass of particles smaller than the indicated Fmin value														
	E 171-P01	**E 171-P02**	**E 171-P03**	**E 171-P04**	**E 171-P05**	**E 171-P06**	**E 171-P07**	**E 171-P09**	**E 171-P15**	**E 171-P16**	**E 171-P17**	**E 171-P18**	**E 171-P19**	**E 171-P20**	**E 171-P21**
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0
60	0	1	1	1	0	1	1	2	0	1	5	1	2	1	1
70	1	2	1	2	0	3	1	5	1	2	11	2	7	1	2
80	2	5	3	4	1	6	3	11	2	5	20	5	13	3	4
90	5	10	6	8	1	12	6	17	5	10	30	9	21	5	9
100	**8**	**16**	**10**	**13**	**3**	**21**	**11**	**23**	**8**	**17**	**41**	**14**	**32**	**8**	**14**
110	12	26	14	18	5	29	17	32	13	21	51	21	42	12	21
120	18	36	20	25	7	38	24	41	20	29	60	29	53	16	28
130	25	44	29	32	11	44	31	46	27	36	68	38	63	22	38
140	32	53	36	40	15	53	40	55	35	40	74	45	71	31	45
150	39	59	44	45	20	58	49	61	44	48	80	51	76	37	54
160	48	66	53	51	25	68	55	67	52	57	84	58	83	44	60
170	55	72	58	58	31	73	61	72	59	66	89	64	87	55	70
180	61	77	64	64	39	76	67	76	64	74	92	72	92	61	76
190	68	81	68	71	48	84	76	82	69	79	94	78	93	63	85
200	72	85	72	77	53	90	82	85	74	86	97	84	96	68	88
250	91	95	88	92	79	100	94	96	93	98	100	95	100	89	98
300	98	100	94	98	93	100	98	96	100	100	100	100	100	100	100
400	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
500	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100