TEST REPORT

Requesting party: FOODWATCH E.V.
Brunnenstrasse 181
10119 BERLIN
Germany

Order date and reference: 01/08/2019 - Agreement on quotation n° 2019/11190

Purpose: Determination of the nano character of an additive (titanium dioxide, TiO₂) in a finished product according to Recommendation EC 2011/696 (Particle size distribution characterisation by SEM and chemical identification by EDX).

This document may only be copied in full.
1. SAMPLES RECEIVED AND INFORMATION RECEIVED

1.1. OBJECTIVE OF THE STUDY

The objective of the study is to determine the number size distribution and the median size of particles of an additive (titanium dioxide, TiO₂) present in a finished product in order to determine if this substance is to be considered as a nanomaterial or not according to EC recommendation on the definition of nanomaterials EC2011/696.

From the received finished product, this will involve:
1) Extracting the additive using a protocol tailored to the consumer product.
2) Preparing the particle populations so as to perform reliable measurements.
3) Identifying the chemical nature of the observed particles using elementary analysis by EDX (Energy-dispersive X-ray spectroscopy).
4) Carrying out measurements on the size, size distribution and shape (qualitative description) of the extracted particles populations by SEM (Scanning Electron Microscopy).

1.2. REMINDER OF DEFINITIONS

- **European Recommendation on the definition of nanomaterials (2011/696/EU)**: "Nanomaterial" means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm".

1.3. SAMPLE RECEIVED

One sample have been received, namely:

- 2 packets of **Dr. Oetker Dekor Kreation Rosa Mix** (Batch L309)

Test report continued on next page
2. EXPERIMENTAL PROTOCOL

2.1. SAMPLE PREPARATION

The preparation of samples for electron microscopy and elementary analysis by EDX depends on the nature of the products to be analysed. The protocol followed will be specified in the Tests section.

2.2. TEST CONDITIONS

- SEM measurements

SEM measurements were performed with a Zeiss Ultra-Plus scanning electron microscope equipped with two secondary electron detectors: SE2 and In-Lens. All images present in this report were obtained with the In-Lens detector.

- Elementary analysis by EDX

Elementary analysis providing information about the atoms constituting the particles is carried out by using the EDX technique. This involves having a detector installed on the electron microscope, which gathers X-ray photons emitted from the particles.

Test report continued on next page
3. TESTS

DR. OETKER DEKOR KREATION ROSA MIX

3.1. PREPARATION OF SAMPLES FOR ELECTRON MICROSCOPY ANALYSIS

Pictures of the studied sample is shown in Figure 1, with the list of ingredients including the mention “E171”, which indicates the presence of titanium dioxide in the product.

![Sample Picture](image)

*Figure 1*: Pictures of the sample “Dr. Oetker Dekor Kreation Rosa Mix”.

The extraction protocol and sample preparation are as follows:

1. A quantity of product (around 500 mg) is mixed with 5 mL ultra-pure Milli-Q water.
2. The obtained suspension is sonicated using an ultrasonic bath.
3. The suspension is then washed by following the steps below:
   1. removal of the supernatant liquid and replacement with hydrochloric acid 1M water,
   2. sonication using a probe sonicator with a power of 150 W,
   3. centrifugation.
   5 successive washes are carried out by repeating these steps.
4. At the end of the 5th wash, the supernatant liquid is replaced with ultra-pure Milli-Q water.
5. The obtained suspension is then redispersed using a probe sonicator.
NP analysis by electron microscopy (SEM) requires specific sample preparation in order to prevent excessive agglomeration of the NPs. To do this, LNE has developed an original protocol involving a spin-coater. This protocol comprises two phases:

1. Spreading a drop of suspension over a silicon substrate with a low rotation speed.

2. Rapid drying of the drop at high rotation speed.

The particles deposited on the silicon substrate are then observed by SEM.
3.2. **SEM MEASUREMENTS AND EDX ANALYSES PERFORMED ON THE EXTRACTED PARTICLES**

Examples of SEM images of the particles observed in the analysed sample are reported on Figure 2. Some plates with micrometric sizes are observed. These plates are made of agglomerated constituent particles with an isotropic shape. Details on notions of constituent particles and agglomerates are given in the annex of the report.

![SEM images of the particles extracted from sample “Dr. Oetker Dekor Kreation Rosa Mix”](image)

*Figure 2: SEM images of the particles extracted from sample “Dr. Oetker Dekor Kreation Rosa Mix”.*

Elementary analysis providing information about the atoms constituting the particles is carried out on the plate of agglomerated constituent particles of Figure 3 by using the EDX technique.

![SEM image of a plate of agglomerated constituent particles extracted from sample “Dr. Oetker Dekor Kreation Rosa Mix”](image)

*Figure 3: SEM image of a plate of agglomerated constituent particles extracted from sample “Dr. Oetker Dekor Kreation Rosa Mix”.*
Figure 4 shows the EDX spectrum of the population of particles constituting the plate of agglomerated constituent particles imaged on Figure 3.

![EDX spectrum](image)

**Figure 4:** EDX spectrum performed on the plate of agglomerated constituent particles imaged on Figure 3.

The peaks relating to the titanium Ti Kα and Ti Kβ lines and to the oxygen O Kα line present in the EDX spectrum above are perfectly visible and clearly indicate the presence of titanium oxide (the silicon Si Kα peak is from the substrate used for the deposition of particles; the carbon C Kα, aluminium Al Kα and potassium K Kα peaks are probably from components of the plate containing these elements).

In order to confirm the chemical composition of the particles, EDX mapping was carried out on the same plate of agglomerated constituent particles. The results are given in Figure 5. The presence of titanium and oxygen corresponds exactly to the location of the particles, which confirms they are titanium dioxide particles.

![EDX mapping](image)

**Figure 5:** EDX mapping performed on the plate of agglomerated constituent particles imaged on Figure 3.
From the SEM images obtained, a set of constituent particles was measured in order to construct a number size distribution histogram. So that the dimensional measurements were representative of the entire population studied, 300 constituent particles were analysed.

![Histogram](image)

\[ D_m = 23.3 \text{ nm} \]
\[ \sigma = 5.3 \text{ nm} \]

*Figure 6: Size distribution histogram of the TiO₂ constituent particles extracted from sample “Dr. Oetker Dekor Kreation Rosa Mix”.*

The histogram is shown in Figure 6 and shows a characteristic normal distribution (red line). The various dimensions drawn from this measurement are collated in the table below.

<table>
<thead>
<tr>
<th>Mesurand</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average size</td>
<td>23.3 nm ± 2.0 nm</td>
</tr>
<tr>
<td>Size distribution (standard deviation)</td>
<td>5.3 nm</td>
</tr>
<tr>
<td>Median size</td>
<td>23.3 nm</td>
</tr>
<tr>
<td>Mode</td>
<td>23.3 nm</td>
</tr>
</tbody>
</table>

*Median size: the size which divides the distribution into two parts of equal area.
Mode: The mode of the distribution is the average size of the most common class.*

From the results of the histogram, 100 % of the constituent particles population by number is less than 100 nm.

Test report continued on next page
4. CONCLUSION

Dr. Oetker Dekor Kreation Rosa Mix:
- The presence of TiO$_2$ particles is confirmed.
- Plates made of agglomerated constituent particles with an isotropic shape are observed.
- The median size of the particles is 23.3 nm.
- 100% of the population of constituent particles by number is less than 100 nm. (i.e. 100% of present particles are nanoparticles).

Trappes, 8 August 2019

Test manager

The results indicated are only applicable to the samples, products or equipment submitted to LNE as they are defined in this document.
Annex

Demonstration of the "nano" character of a particle-state substance - General elements of understanding

The term "nanomaterial" can mean substances that are natural (volcanic ash, etc.), anthropogenic (transport, etc.) or intentionally manufactured on this scale to take advantage of:

- either new properties which appear specifically in this size range,
- or increased surface to volume ratio and an associated increased reactivity as the particle size tends to decrease.

These two specific characteristics of nanomaterials are directly responsible for the interest focussed today on these substances by all industrial sectors (health, energy, food, construction, etc.). Within the food sector or cosmetic product sector, nanomaterials are used as additives in the products themselves (E551 = amorphous silica, E171 = titanium dioxide TiO₂, etc.) or for packaging materials (nanoclay, nanoAg), etc. in order to improve various properties. Some of them, such as E551, have even been used for decades as anti-caking agents in powder products.

Several studies, however, have shown that nanomaterials, and notably nanoparticles, have a toxicity which is clearly different from macroscopic materials of the same composition, which means that studies are required on risk assessments linked to these new materials. The fact that nanomaterials are defined by a large number of parameters, as reported in document ISO TS 13014 (size, size distribution, shape, agglomeration/aggregation state, etc.). does not however enable general rules to be currently applied as to their performance and possible toxicity. This reflects the importance of having exhaustive characterisation data and positions nanometrology as the basis for better control of manufacturing processes, improvement of quality systems and risk assessments linked to these new materials.

The specific characteristics of nanomaterials have therefore been taken into consideration in European regulatory texts (Cosmetic regulation No. 1223/2009), INCO Regulation No. 1169/2011, Biocide Regulation No. 528/2012, etc.) or French regulatory texts (Decree No. 2012-232 and Order of 6 August 2012). Among the various requirements associated with these texts, companies are notably required to attach to the ingredient concerned the wording [nano] on the product label in order to inform the consumer. This means there is a requirement to be able to determine in a reliable way the nano character of the targeted particle-state substances.

Various definitions of nanomaterials currently co-exist in Europe with certain particularities, such as:

- intentionally-manufactured, with a size of less than 100 nm OR greater than 100 nm BUT with specific nanoscale properties, in the Novel Food Regulation No. 2283/2;
- intentionally manufactured + insolubility and size less than 100 nm, in Cosmetic Regulation No. 1223/2009.

In order to harmonise what is meant by nanomaterials and to avoid a substance being considered nano in one sector but not in another, in 2011, the European Commission proposed a Recommendation on the Definition (2011/696/EU):

"Nanomaterial" means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm."
The demonstration of the nano character within the meaning of the Recommendation on the Definition 2011/696/EU therefore requires a determination of the particle size distribution of the population of constituent particles (free or constituting agglomerates or aggregates present) and to extract from them the median diameter corresponding to the 50% threshold. The need to access the size of the constituent particles is justified in Recommendation 2011/696/EU by the fact that throughout the life-cycle of a nanomaterial, particles can become detached from an agglomerate or aggregate: "The definition in this recommendation should therefore also include particles in agglomerates or aggregates, whenever the constituent particles are in the size range 1 nm - 100 nm" (point 12). The JRC document « An overview of concepts and terms used in the European Commission’s definition of nanomaterial » reviews concepts and terms used in the Recommendation 2011/696/EU (http://publications.jrc.ec.europa.eu/repository/bitstream/JRC113469/kjna29647enn.pdf).

Numerous techniques enable the size distribution of a sample to be characterised (DLS, A4F-MALS, sp-ICPMS, CLS, SMPS, NTA, SEM, TEM, AFM, etc.), but to date, none of them is perfect. Completely different results may thus be obtained depending on the technique used as the associated measurement will not necessarily be the same, as was demonstrated by an inter-laboratory comparison organised within the scope of the Club nanoMetrologie (www.club-nanometrologie.fr). Each one has its own specific limitations (low accessible size limit, confusion between nanoparticle agglomerates/aggregates and larger constituent particles, etc.) and many of them cannot therefore be used in an initial screening step (DLS, CLS, sp-ICPMS, etc.). It is therefore recommended to combine results obtained via several of these analytical techniques in order to ensure that the information retrieved is reliable.

In order to access quantitative and reliable data, it is necessary to use SEM (Scanning Electron Microscopy) or TEM (Transmission Electron Microscopy) microscopy techniques. A high degree of expertise in sample preparation and interpretation of the images obtained is however necessary in order to extract high quality results. These conclusions are notably those given by the flagship project by the European Commission on this subject (NanoDefine project, www.nanodeline.eu) and the NANOMET project (www.nanomet.fr) financed in France by the DGE. The LNE participates in these two research projects and has thus developed reference methodologies over recent years in order to ensure that the various key steps in the measurement process are reliable:

- sample preparation,
- instrument calibration,
- data acquisition protocol,
- data processing and measurement uncertainty evaluation.

The application of ultrasound and adjustment of the pH during the sample preparation phase enable agglomerates present to be broken down and thus enables the constituent particle size to be characterised more reliably.

Recommendation 2011/696/EU also specifies that "Where technically feasible and required under specific legislation, compliance with the definition ... may be determined on the basis of the volume-specific surface area (VSSA). A material is to be considered relevant to the definition ... where the volume-specific surface area of the material is greater than 60 m²/cm³." The volume-specific surface area is determined via the SEM technique and the threshold of 60 m²/cm³ corresponds to a population of spherical, mono-dispersed and non-porous particles 100 nm in size.
Note

LNE implements its cutting-edge instrumentation and reference protocols developed during this research work as the French National Metrology Laboratory in order to determine the "nano" character of particle-state substances and to provide reliable results.

Its expertise focuses on measurements and metrology and under no circumstances on toxicity issues potentially associated with these substances.
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1. SAMPLES RECEIVED AND INFORMATION RECEIVED

1.1. OBJECTIVE OF THE STUDY

The objective of the study is to determine the number size distribution and the median size of particles of an additive (titanium dioxide, TiO₂) present in a finished product in order to determine if this substance is to be considered as a nanomaterial or not according to EC recommendation on the definition of nanomaterials EC2011/696.

From the received finished product, this will involve:
1) Extracting the additive using a protocol tailored to the consumer product.
2) Preparing the particle populations so as to perform reliable measurements.
3) Identifying the chemical nature of the observed particles using elementary analysis by EDX (Energy-dispersive X-ray spectroscopy).
4) Carrying out measurements on the size, size distribution and shape (qualitative description) of the extracted particles populations by SEM (Scanning Electron Microscopy).

1.2. REMINDER OF DEFINITIONS

- **European Recommendation on the definition of nanomaterials (2011/696/EU):** "Nanomaterial" means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm".

1.3. SAMPLE RECEIVED

One sample have been received, namely:

2 packets of **Dr. Oetker Streusel Kuchen** (Batch L102)

Test report continued on next page
2. EXPERIMENTAL PROTOCOL

2.1. Sample preparation

The preparation of samples for electron microscopy and elementary analysis by EDX depends on the nature of the products to be analysed. The protocol followed will be specified in the Tests section.

2.2. Test conditions

- SEM measurements

SEM measurements were performed with a Zeiss Ultra-Plus scanning electron microscope equipped with two secondary electron detectors: SE2 and In-Lens. All images present in this report were obtained with the In-Lens detector.

- Elementary analysis by EDX

Elementary analysis providing information about the atoms constituting the particles is carried out by using the EDX technique. This involves having a detector installed on the electron microscope, which gathers X-ray photons emitted from the particles.
3. TESTS

DR. OETKER STREUSEL KUCHEN

3.1. PREPARATION OF SAMPLES FOR ELECTRON MICROSCOPY ANALYSIS

A picture of the studied sample is shown in Figure 1, with the list of ingredients including the mention "Titandioxid", which indicates the presence of titanium dioxide in the product. The product is made of two parts: "Mischung für den Belag" ("Cremeblending") and "Backmischung" ("Bagelblanding"). The "Cremeblanding" part has been analysed for the presence and characterization of titanium dioxide particles.

![Figure 1: Pictures of the sample "Dr. Oetker Streusel Kuchen".](image)

The extraction protocol and sample preparation are as follows:

1. A quantity of "Cremeblending" (around 150 mg) is mixed with 10 mL ultra-pure Milli-Q water.
2. The obtained suspension is sonicated using an ultrasonic bath.
3. The suspension is then centrifugated.
4. The suspension is then washed with ultra-pure Milli-Q water by following the steps below:
   (1) removal of the supernatant liquid and replacement with ultra-pure Milli-Q water,
   (2) sonication using a probe sonicator with a power of 150 W,
   (3) centrifugation.
5 successive washes are carried out by repeating these steps.

5. The suspension is then washed with ethanol by following the steps below:
   (1) removal of the supernatant liquid and replacement with ethanol,
   (2) sonication using a probe sonicator with a power of 150 W,
   (3) centrifugation.

6. The suspension is then washed with acetone by following the steps below:
   (1) removal of the supernatant liquid and replacement with acetone,
   (2) sonication using a probe sonicator with a power of 150 W,
   (3) centrifugation.
   5 successive washes are carried out by repeating these steps.

7. At the end of the 5th wash with acetone, the supernatant liquid is replaced by acetone.

8. The obtained suspension is then redispersed using a probe sonicator.

NP analysis by electron microscopy (SEM) requires specific sample preparation in order to prevent excessive agglomeration of the NPs. To do this, LNE has developed an original protocol involving a spin-coater. This protocol comprises two phases:

1. Spreading a drop of suspension over a silicon substrate with a low rotation speed.

2. Rapid drying of the drop at high rotation speed.

The particles deposited on the silicon substrate are then observed by SEM.
3.2. **SEM measurements and EDX analyses performed on the extracted particles**

Examples of SEM images of the particles observed in the analysed sample are reported on Figure 2. Some agglomerates made of constituent particles with an isotropic shape are observed. These agglomerates have variable sizes. The shape and the size of the extracted particles are typical of TiO₂. Details on notions of constituent particles and agglomerates are given in the annex of the report.

![Figure 2: SEM images of the particles extracted from sample "Dr. Oetker Streusel Kuchen".](image)

Elementary analysis providing information about the atoms constituting the particles is carried out on the agglomerate of Figure 3 by using the EDX technique.

![Figure 3: SEM image of an agglomerate of constituent particles extracted from sample "Dr. Oetker Streusel Kuchen".](image)
Figure 4 shows the EDX spectrum of the population of particles constituting the agglomerate imaged on Figure 3.

![EDX Spectrum](image)

*Figure 4: EDX spectrum performed on the agglomerate of constituent particles imaged on Figure 3.*

The peaks relating to the titanium Ti Kα and Ti Kβ lines and to the oxygen O Kα line present in the EDX spectrum above are perfectly visible and clearly indicate the presence of titanium oxide (the silicon Si Kα peak is from the substrate used for the deposition of particles; the carbon C Kα peak is probably from residues of food matrix).

In order to confirm the chemical composition of the particles, EDX mapping was carried out on the same agglomerate. The results are given in Figure 5. The presence of titanium and oxygen corresponds exactly to the location of the particles, which confirms they are titanium dioxide particles.

![EDX Mapping](image)

*Figure 5: EDX mapping performed on the agglomerate imaged on Figure 3.*
From the SEM images obtained, a set of constituent particles was measured in order to construct a number size distribution histogram. So that the dimensional measurements were representative of the entire population studied, 300 constituent particles were analysed.

\[ D_m = 135.3 \text{ nm} \]
\[ \sigma = 47.8 \text{ nm} \]

*Figure 6: Size distribution histogram of the TiO\(_2\) constituent particles extracted from sample "Dr. Oetker Streusel Kuchen".*

The histogram is shown in Figure 6 and shows a characteristic log-normal distribution (red line). The various dimensions drawn from this measurement are collated in the table below.

<table>
<thead>
<tr>
<th>Mesurand</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Average size</td>
<td>135.3 nm ± 2.7 nm</td>
</tr>
<tr>
<td>Size distribution (standard deviation)</td>
<td>47.8 nm</td>
</tr>
<tr>
<td>Median size</td>
<td>127.6 nm</td>
</tr>
<tr>
<td>Mode</td>
<td>113.5 nm</td>
</tr>
</tbody>
</table>

Median size: the size which divides the distribution into two parts of equal area. Mode: The mode of the distribution is the average size of the most common class.

From the results of the histogram, 22 % of the constituent particles population by number is less than 100 nm.
4. CONCLUSION

Dr. Oetker Streusel Kuchen:

- The presence of TiO$_2$ particles is confirmed.
- Agglomerates made of constituent particles with an isotropic shape are observed.
- The median size of the particles is 127.6 nm.
- 22% of the population of constituent particles by number is less than 100 nm. (i.e. 22% of present particles are nanoparticles).

Trappes, 8 August 2019

Test manager

The results indicated are only applicable to the samples, products or equipment submitted to LNE as they are defined in this document.
Annex

Demonstration of the "nano" character of a particle-state substance - General elements of understanding

The term "nanomaterial" can mean substances that are natural (volcanic ash, etc.), anthropogenic (transport, etc.) or intentionally manufactured on this scale to take advantage of:

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These two specific characteristics of nanomaterials are directly responsible for the interest focussed today on these substances by all industrial sectors (health, energy, food, construction, etc.). Within the food sector or cosmetic product sector, nanomaterials are used as additives in the products themselves (E551 = amorphous silica, E171= titanium dioxide TiO₂, etc.) or for packaging materials (nanoclay, nanoAg), etc. in order to improve various properties. Some of them, such as E551, have even been used for decades as anti-caking agents in powder products.

Several studies, however, have shown that nanomaterials, and notably nanoparticles, have a toxicity which is clearly different from macromolecular substances of the same composition, which means that studies are required on risk assessments linked to these new materials. The fact that nanomaterials are defined by a large number of parameters, as reported in document ISO TS 13014 (size, size distribution, shape, agglomeration/aggregation state, etc.), does not however enable general rules to be currently applied as to their performance and possible toxicity. This reflects the importance of having exhaustive characterisation data and positions nanometrology as the basis for better control of manufacturing processes, improvement of quality systems and risk assessments linked to these new materials.

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LNE
The demonstration of the nano character within the meaning of the Recommendation on the Definition 2011/696/EU therefore requires a determination of the particle size distribution of the population of constituent particles (free or constituting agglomerates or aggregates present) and to extract from them the median diameter corresponding to the 50% threshold. The need to access the size of the constituent particles is justified in Recommendation 2011/696/EU by the fact that throughout the life-cycle of a nanomaterial, particles can become detached from an agglomerate or aggregate: "The definition in this recommendation should therefore also include particles in agglomerates or aggregates, whenever the constituent particles are in the size range 1 nm - 100 nm" (point 12). The JRC document « An overview of concepts and terms used in the European Commission's definition of nanomaterial » reviews concepts and terms used in the Recommendation 2011/696/EU (http://publications.jrc.ec.europa.eu/repository/bitstream/JRC113469/kjina29647enn.pdf).

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1.3. SAMPLE RECEIVED

One sample have been received, namely:

2 packets of **Dr. Oetker Lustige Zuckeraugen** (Batch L8243)

Test report continued on next page
2. EXPERIMENTAL PROTOCOL

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3. TESTS

DR. OETKER LUSTIGE ZUCKERAUGEN

3.1. PREPARATION OF SAMPLES FOR ELECTRON MICROSCOPY ANALYSIS

A picture of the studied sample is shown in Figure 1, with the list of ingredients including the mention "E171", which indicates the presence of titanium dioxide in the product.

![Image of Dr. Oetker Lustige Zuckeraugen](image)

*Figure 1: Pictures of the sample "Dr. Oetker Lustige Zuckeraugen".*

The extraction protocol and sample preparation are as follows:

1. One "sugar eye" (around 500 mg) is mixed with 10 mL ultra-pure Milli-Q water.
2. The obtained suspension is sonicated using an ultrasonic bath.
3. The suspension is then washed by following the steps below:
   (1) removal of the supernatant liquid and replacement with ultra-pure Milli-Q water,
   (2) sonication using a probe sonicator with a power of 150 W,
   (3) centrifugation.
   5 successive washes are carried out by repeating these steps.
4. At the end of the 5th wash, the supernatant liquid is replaced with ultra-pure Milli-Q water.
5. The obtained suspension is then redispersed using a probe sonicator.
NP analysis by electron microscopy (SEM) requires specific sample preparation in order to prevent excessive agglomeration of the NPs. To do this, LNE has developed an original protocol involving a spin-coater. This protocol comprises two phases:

1. Spreading a drop of suspension over a silicon substrate with a low rotation speed.

2. Rapid drying of the drop at high rotation speed.

The particles deposited on the silicon substrate are then observed by SEM.
3.2. SEM MEASUREMENTS AND EDX ANALYSES PERFORMED ON THE EXTRACTED PARTICLES

Examples of SEM images of the particles observed in the analysed sample are reported on Figure 2. Some agglomerates made of constituent particles with an isotropic shape are observed. These agglomerates have sizes of several hundred nanometers. The shape and the size of the extracted particles are typical of TiO₂. Details on notions of constituent particles and agglomerates are given in the annex of the report.

Figure 2: SEM images of the particles extracted from sample “Dr. Oetker Lustige Zuckeraugen”.

Elementary analysis providing information about the atoms constituting the particles is carried out on the agglomerate of Figure 3 by using the EDX technique.

Figure 3: SEM image of an agglomerate of constituent particles extracted from sample “Dr. Oetker Lustige Zuckeraugen”.
Figure 4 shows the EDX spectrum of the population of particles constituting the agglomerate imaged on Figure 3.

![EDX spectrum](image)

**Figure 4:** EDX spectrum performed on the agglomerate of constituent particles imaged on Figure 3.

The peaks relating to the titanium Ti $K\alpha$ and Ti $K\beta$ lines and to the oxygen O $K\alpha$ line present in the EDX spectrum above are perfectly visible and clearly indicate the presence of titanium oxide (the silicon Si $K\alpha$ peak is from the substrate used for the deposition of particles; the carbon C $K\alpha$ peak is probably from residues of food matrix).

In order to confirm the chemical composition of the particles, EDX mapping was carried out on the same agglomerate. The results are given in Figure 5. The presence of titanium and oxygen corresponds exactly to the location of the particles, which confirms they are titanium dioxide particles.

![EDX mapping](image)

**Figure 5:** EDX mapping performed on the agglomerate imaged on Figure 3.

Test report continued on next page
From the SEM images obtained, a set of constituent particles was measured in order to construct a number size distribution histogram. So that the dimensional measurements were representative of the entire population studied, 300 constituent particles were analysed.

![Histogram of TiO2 constituent particles](image)

*Figure 6: Size distribution histogram of the TiO2 constituent particles extracted from sample “Dr. Oetker Lustige Zuckeraugen”.

The histogram is shown in Figure 6 and shows a characteristic log-normal distribution (red line). The various dimensions drawn from this measurement are collated in the table below. The various dimensions drawn from this measurement are collated in the table below.

<table>
<thead>
<tr>
<th>Measurand</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average size</td>
<td>124.0 nm ± 2.5 nm</td>
</tr>
<tr>
<td>Size distribution (standard deviation)</td>
<td>46.5 nm</td>
</tr>
<tr>
<td>Median size</td>
<td>116.1 nm</td>
</tr>
<tr>
<td>Mode</td>
<td>101.8 nm</td>
</tr>
</tbody>
</table>

Median size: the size which divides the distribution into two parts of equal area.

Mode: The mode of the distribution is the average size of the most common class.

From the results of the histogram, 33% of the constituent particles population by number is less than 100 nm.
4. CONCLUSION

Dr. Oetker Lustige Zuckeraugen:

- The presence of TiO\textsubscript{2} particles is confirmed.
- Agglomerates made of constituent particles with an isotropic shape are observed.
- The median size of the particles is 116.1 nm.
- 33 % of the population of constituent particles by number is less than 100 nm. (i.e. 33 % of present particles are nanoparticles).

Trappes, 8 August 2019

Test manager

The results indicated are only applicable to the samples, products or equipment submitted to LNE as they are defined in this document.
Annex

Demonstration of the "nano" character of a particle-state substance - General elements of understanding

The term "nanomaterial" can mean substances that are natural (volcanic ash, etc.), anthropogenic (transport, etc.) or intentionally manufactured on this scale to take advantage of:

- either new properties which appear specifically in this size range,
- or increased surface to volume ratio and an associated increased reactivity as the particle size tends to decrease.

These two specific characteristics of nanomaterials are directly responsible for the interest focused today on these substances by all industrial sectors (health, energy, food, construction, etc.). Within the food sector or cosmetic product sector, nanomaterials are used as additives in the products themselves (E551 = amorphous silica, E171 = titanium dioxide TiO₂, etc.) or for packaging materials (nanoclay, nanoAg), etc. in order to improve various properties. Some of them, such as E551, have even been used for decades as anti-caking agents in powder products.

Several studies, however, have shown that nanomaterials, and notably nanoparticles, have a toxicity which is clearly different from macroscopic materials of the same composition, which means that studies are required on risk assessments linked to these new materials. The fact that nanomaterials are defined by a large number of parameters, as reported in document ISO TS 13014 (size, size distribution, shape, agglomeration/aggregation state, etc.), does not however enable general rules to be currently applied as to their performance and possible toxicity. This reflects the importance of having exhaustive characterisation data and positions nanometrology as the basis for better control of manufacturing processes, improvement of quality systems and risk assessments linked to these new materials.

The specific characteristics of nanomaterials have therefore been taken into consideration in European regulatory texts (Cosmetic regulation No. 1223/2009, INCO Regulation No. 1169/2011, Biocide Regulation No. 528/2012, etc.) or French regulatory texts (Decree No. 2012-232 and Order of 6 August 2012). Among the various requirements associated with these texts, companies are notably required to attach to the ingredient concerned the wording [nano] on the product label in order to inform the consumer. This means there is a requirement to be able to determine in a reliable way the nano character of the targeted particle-state substances.

Various definitions of nanomaterials currently co-exist in Europe with certain particularities, such as:

- intentionally-manufactured, with a size of less than 100 nm OR greater than 100 nm BUT with specific nanoscale properties, in the Novel Food Regulation No. 2283/2;
- intentionally manufactured + insolubility and size less than 100 nm, in Cosmetic Regulation No. 1223/2009.

In order to harmonise what is meant by nanomaterials and to avoid a substance being considered nano in one sector but not in another, in 2011, the European Commission proposed a Recommendation on the Definition (2011/696/EU):

"Nanomaterial" means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm."
The demonstration of the nano character within the meaning of the Recommendation on the Definition 2011/696/EU therefore requires a determination of the particle size distribution of the population of constituent particles (free or constituting agglomerates or aggregates present) and to extract from them the median diameter corresponding to the 50% threshold. The need to access the size of the constituent particles is justified in Recommendation 2011/696/EU by the fact that throughout the life-cycle of a nanomaterial, particles can become detached from an agglomerate or aggregate: "The definition in this recommendation should therefore also include particles in agglomerates or aggregates, whenever the constituent particles are in the size range 1 nm - 100 nm" (point 12). The JRC document « An overview of concepts and terms used in the European Commission’s definition of nanomaterial » reviews concepts and terms used in the Recommendation 2011/696/EU (http://publications.jrc.ec.europa.eu/repository/bitstream/JRC111349/kina29647en.pdf).

Numerous techniques enable the size distribution of a sample to be characterised (DLS, A4F-MALS, sp-ICPMS, CLS, SMPS, NTA, SEM, TEM, AFM, etc.), but to date, none of them is perfect. Completely different results may thus be obtained depending on the technique used as the associated measurement will not necessarily be the same, as was demonstrated by an inter-laboratory comparison organised within the scope of the Club nanoMetrologie (www.club-nanometrologie.fr). Each one has its own specific limitations (low accessible size limit, confusion between nanoparticle agglomerates/aggregates and larger constituent particles, etc.) and many of them cannot therefore be used in an initial screening step (DLS, CLS, sp-ICPMS, etc.). It is therefore recommended to combine results obtained via several of these analytical techniques in order to ensure that the information retrieved is reliable.

In order to access quantitative and reliable data, it is necessary to use SEM (Scanning Electron Microscopy) or TEM (Transmission Electron Microscopy) microscopy techniques. A high degree of expertise in sample preparation and interpretation of the images obtained is however necessary in order to extract high quality results. These conclusions are notably those given by the flagship project by the European Commission on this subject (NanoDefine project, www.nanodelinee.eu) and the NANOMET project (www.nanomet.fr) financed in France by the DGE. The LNE participates in these two research projects and has thus developed reference methodologies over recent years in order to ensure that the various key steps in the measurement process are reliable:

- sample preparation,
- instrument calibration,
- data acquisition protocol,
- data processing and measurement uncertainty evaluation.

The application of ultrasound and adjustment of the pH during the sample preparation phase enable agglomerates present to be broken down and thus enables the constituent particle size to be characterised more reliably.

Recommendation 2011/696/EU also specifies that "Where technically feasible and required under specific legislation, compliance with the definition ... may be determined on the basis of the volume-specific surface area (VSSA). A material is to be considered relevant to the definition ... where the volume-specific surface area of the material is greater than 60 m^2/cm^3. " The volume-specific surface area is determined via the SEM technique and the threshold of 60 m^2/cm^3 corresponds to a population of spherical, mono-dispersed and non-porous particles 100 nm in size.
Note

LNE implements its cutting-edge instrumentation and reference protocols developed during this research work as the French National Metrology Laboratory in order to determine the "nano" character of particle-state substances and to provide reliable results.

Its expertise focusses on measurements and metrology and under no circumstances on toxicity issues potentially associated with these substances.
TEST REPORT

Requesting party: FOODWATCH E.V.
Brunnenstrasse 181
10119 BERLIN
Germany

Order date and reference: 01/08/2019 - Agreement on quotation n° 2019/11190

Purpose: Determination of the nano character of an additive (titanium dioxide, TiO₂) in a finished product according to Recommendation EC 2011/696 (Particle size distribution characterisation by SEM and chemical identification by EDX).

This document may only be copied in full.
1. SAMPLES RECEIVED AND INFORMATION RECEIVED

1.1. OBJECTIVE OF THE STUDY

The objective of the study is to determine the number size distribution and the median size of particles of an additive (titanium dioxide, TiO₂) present in a finished product in order to determine if this substance is to be considered as a nanomaterial or not according to EC recommendation on the definition of nanomaterials EC2011/696.

From the received finished product, this will involve:
1) Extracting the additive using a protocol tailored to the consumer product.
2) Preparing the particle populations so as to perform reliable measurements.
3) Identifying the chemical nature of the observed particles using elementary analysis by EDX (Energy-dispersive X-ray spectroscopy).
4) Carrying out measurements on the size, size distribution and shape (qualitative description) of the extracted particles populations by SEM (Scanning Electron Microscopy).

1.2. REMINDER OF DEFINITIONS

- European Recommendation on the definition of nanomaterials (2011/696/EU): “Nanomaterial” means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm”.

1.3. SAMPLE RECEIVED

One sample have been received, namely:

2 packets of Dr. Oetker Fix & Fertig Zuckerguss Classic (Batch L339)
2. EXPERIMENTAL PROTOCOL

2.1. SAMPLE PREPARATION

The preparation of samples for electron microscopy and elementary analysis by EDX depends on the nature of the products to be analysed. The protocol followed will be specified in the Tests section.

2.2. TEST CONDITIONS

- SEM measurements

SEM measurements were performed with a Zeiss Ultra-Plus scanning electron microscope equipped with two secondary electron detectors: SE2 and In-Lens. All images present in this report were obtained with the In-Lens detector.

- Elementary analysis by EDX

Elementary analysis providing information about the atoms constituting the particles is carried out by using the EDX technique. This involves having a detector installed on the electron microscope, which gathers X-ray photons emitted from the particles.

Test report continued on next page
3. TESTS

DR. OETKER FIX & FERTIG ZUCKERGUSS CLASSIC

3.1. PREPARATION OF SAMPLES FOR ELECTRON MICROSCOPY ANALYSIS

Pictures of the studied sample is shown in Figure 1, with the list of ingredients including the mention “E171”, which indicates the presence of titanium dioxide in the product.

![Package of Dr. Oetker Fix & Fertig Zuckergruss Classic](image)

*Figure 1: Pictures of the sample “Dr. Oetker Fix & Fertig Zuckergruss Classic”.*

The extraction protocol and sample preparation are as follows:

1. A quantity of product (around 200 mg) is mixed with 10 mL ultra-pure Milli-Q water.
2. The obtained suspension is sonicated using an ultrasonic bath.
3. The suspension is then washed by following the steps below:
   1. removal of the supernatant liquid and replacement with ultra-pure Milli-Q water,
   2. sonication using a probe sonicator with a power of 150 W,
   3. centrifugation.
   5 successive washes are carried out by repeating these steps.
4. At the end of the 5th wash, the supernatant liquid is replaced with ultra-pure Milli-Q water.
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NP analysis by electron microscopy (SEM) requires specific sample preparation in order to prevent excessive agglomeration of the NPs. To do this, LNE has developed an original protocol involving a spin-coater. This protocol comprises two phases:

1. Spreading a drop of suspension over a silicon substrate with a low rotation speed.

2. Rapid drying of the drop at high rotation speed.

The particles deposited on the silicon substrate are then observed by SEM.

Test report continued on next page
3.2. **SEM MEASUREMENTS AND EDX ANALYSES PERFORMED ON THE EXTRACTED PARTICLES**

Examples of SEM images of the particles observed in the analysed sample are reported on Figure 2. Some agglomerates made of constituent particles with an isotropic shape are observed. These agglomerates have variable sizes. The shape and the size of the extracted particles are typical of TiO₂. Details on notions of constituent particles and agglomerates are given in the annex of the report.

![Figure 2: SEM images of the particles extracted from sample "Dr. Oetker Fix & Fertig Zuckerguss Classic".](image)

Elementary analysis providing information about the atoms constituting the particles is carried out on the agglomerate of Figure 3 by using the EDX technique.

![Figure 3: SEM image of an agglomerate of constituent particles extracted from sample "Dr. Oetker Fix & Fertig Zuckerguss Classic".](image)
Figure 4 shows the EDX spectrum of the population of particles constituting the agglomerate imaged on Figure 3.

![EDX Spectrum]

*Figure 4: EDX spectrum performed on the agglomerate of constituent particles imaged on Figure 3.*

The peaks relating to the titanium Ti Kα and Ti Kβ lines and to the oxygen O Kα line present in the EDX spectrum above are perfectly visible and clearly indicate the presence of titanium oxide (the silicon Si Kα peak is from the substrate used for the deposition of particles).

In order to confirm the chemical composition of the particles, EDX mapping was carried out on the same agglomerate. The results are given in Figure 5. The presence of titanium and oxygen corresponds exactly to the location of the particles, which confirms they are titanium dioxide particles.

![EDX Mapping]

*Figure 5: EDX mapping performed on the agglomerate imaged on Figure 3.*
From the SEM images obtained, a set of constituent particles was measured in order to construct a number size distribution histogram. So that the dimensional measurements were representative of the entire population studied, 300 constituent particles were analysed.

![Histogram](image)

*D_{n} = 117.3 \text{ nm}  \quad \sigma = 49.9 \text{ nm}

*Figure 6: Size distribution histogram of the TiO_{2} constituent particles extracted from sample "Dr. Oetker Fix & Fertlig Zuckergruss Classic".*

The histogram is shown in Figure 6 and shows a characteristic log-normal distribution (red line). The various dimensions drawn from this measurement are collated in the table below.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average size</td>
<td>117.3 nm ± 2.4 nm</td>
</tr>
<tr>
<td>Size distribution (standard deviation)</td>
<td>49.9 nm</td>
</tr>
<tr>
<td>Median size</td>
<td>107.9 nm</td>
</tr>
<tr>
<td>Mode</td>
<td>91.4 nm</td>
</tr>
</tbody>
</table>

*Median size: the size which divides the distribution into two parts of equal area. Mode: The mode of the distribution is the average size of the most common class.*

From the results of the histogram, 42 % of the constituent particles population by number is less than 100 nm.

Test report continued on next page
4. CONCLUSION

Dr. Oetker Fix & Fertig Zuckerguss Classic:

- The presence of TiO$_2$ particles is confirmed.
- Agglomerates made of constituent particles with an isotropic shape are observed.
- The median size of the particles is 107.9 nm.
- 42% of the population of constituent particles by number is less than 100 nm. (i.e. 42% of present particles are nanoparticles).

Trappes, 8 August 2019

Test manager

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Annex

Demonstration of the "nano" character of a particle-state substance - General elements of understanding

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Several studies, however, have shown that nanomaterials, and notably nanoparticles, have a toxicity which is clearly different from macroscopic materials of the same composition, which means that studies are required on risk assessments linked to these new materials. The fact that nanomaterials are defined by a large number of parameters, as reported in document ISO TS 13014 (size, size distribution, shape, agglomeration/aggregation state, etc.), does not however enable general rules to be currently applied as to their performance and possible toxicity. This reflects the importance of having exhaustive characterisation data and positions nanometrology as the basis for better control of manufacturing processes, improvement of quality systems and risk assessments linked to these new materials.

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