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Characterisation of titanium dioxide nanoparticles in food products: Analytical methods to define nanoparticles

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Abstract

Titanium dioxide (TiO_2) is a common food additive used to enhance the white colour, brightness and sometimes flavour of a variety of food products. In this study seven food grade TiO_2 materials (E171), 24 food products and three personal care products were investigated for their TiO_2 content and the number-based size distribution of TiO_2 particles present in these products. Three principally different methods have been used to determine the number-based size distribution of TiO_2 particles: electron microscopy, asymmetric flow field-flow fractionation combined with inductively coupled mass spectrometry and single-particle inductively coupled mass spectrometry. The results show that all E171 materials have similar size distributions with primary particle sizes in the range of 60 to 300 nm. Depending on the analytical method used, 10-15% of the particles in these materials had sizes below 100 nm. In 24 of the 27 foods and personal care products detectable amounts of titanium were found ranging from 0.02 to 9.0 mg TiO_2/g product. The number-based size distributions for TiO_2 particles in the food and personal care products showed that 5-10% of the particles in these products had sizes below 100 nm, comparable to that found in the E171 materials. Comparable size distributions were found using the three principally different analytical methods. While the applied methods are considered state of the art, they showed practical size limits for TiO_2 particles in the range of 20 to 50 nm which may introduce a significant bias in the size distribution since particles <20 nm are excluded. This shows the inability of current state of the art methods to support the EU recommendation for the definition of nanomaterials.

Keywords

Titanium dioxide, food additive, nanomaterial, AF4, ICP-MS, single particle ICP-MS

Introduction

Titanium dioxide (TiO_2) is a naturally occurring oxide of the element titanium also referred to as titania. This substance occurs naturally as three mineral compounds known as anatase, rutile and brookite. There are a number of industrial applications for this mineral because of its very high refraction properties. In fact, TiO_2 is one of the whitest materials known to exist, hence the name "titanium white". For this reason, it is often included in cosmetic preparations and sunblock's to reflect light away from the skin. It is also incorporated in paints and in a number of construction and building materials. Scattering of light by TiO_2 is maximized in particles that are 200–300 nm in diameter, and most commercial products that are used as pigments have primary particle sizes within this range *(1)*. The size distribution of ultrasonically dispersed primary particles and aggregates generally ranges from <100 to 500 nm *(2, 3, 4)*. Ultrafine TiO_2 particles range in size from 1 to 150 nm with a mean primary particle size of 10–50 nm *(3)*. These ultrafine TiO_2 particles are mainly used for special applications such as photo catalysts. Since the refractive power of TiO_2 particles is higher at the nanoscale, many applications of TiO_2 such as sunscreens, cosmetics, coatings and especially photo catalytic applications would benefit from smaller primary particle sizes and therefore it is expected that the percentage of TiO_2 that is produced in or near the nano-size range will increase *(5, 6)*.

TiO_2 is commonly used as food additive, and is authorized for its use in the EU as E171 *(7)*. As a pigment, TiO_2 is used to enhance the white colour of certain foods, such as dairy products and candy. It also lends brightness to toothpaste and some medications. However, it is also used as a food additive and flavour enhancer in a variety of non-white foods, including dried vegetables, nuts, seeds, soups, and mustard, as well as beer and wine *(8)*.

In recent years concerns have been raised with regard to the toxicity of nanosized TiO_2 following oral exposure. Five day exposure to 1 or 2 mg/kg body weight (bw) per day anatase TiO_2 resulted in significantly increased Ti levels in ovary and spleen and is suggestive of hormonal effects. The primary particle size of these TiO_2 NP was <25 nm which agglomerated into materials of which 13% of the particles was smaller than 100 nm. The size distribution was dominated by agglomerates with a mean diameter up to 1.6 µm *(9)*. Further, a 90 day intragastric exposure to 2.5, 5 and 10 mg/kg anatase TiO_2 nanoparticles with a crystallite size of 5.5 nm and a hydrodynamic size around 300 nm (in an 0.5% hydroxypropylmethylcellulose solvent) resulted in several kidney effects (renal inflammation, tissue necrosis and renal apoptosis *(10)*. In addition the International Agency for Research on Cancer (IARC) has now classified nano-sized TiO_2 as a potential carcinogen based on the rate of incidence of respiratory tract cancer in rats after prolonged inhalation of TiO_2 dust particles *(11)*. This indicates the need to evaluate the possible carcinogenicity of nano-sized TiO_2 following other exposure routes.

A Monte Carlo human exposure analysis indicated that US adults are exposed to about 1 mg Ti per kg bw per day *(8)*. These authors indicate that in the assessed food and consumer products approximately 36% of the TiO_2 particles were less than 100 nm in one dimension. While TiO2 has been authorized as food additive *(7)* no recent risk assessment is available. In general the risk assessment of oral exposure to nanoparticles is difficult because of a number of uncertainties and a general lack of data *(12)*. Specifically for TiO_2, knowledge about the particle sizes and size distribution of TiO_2 particles in E171, let alone food and consumer products containing E171 is limited. The European Commission adopted a recommendation for the definition of nanomaterials, Commission Recommendation 2011/696/EU states that a material is a nanomaterial if 50% or more of the particles in a number-based size distribution, has one or more external dimensions in the size range of 1-100 nm. In specific cases the number-based size distribution threshold of 50% may be replaced by a threshold between 1 and 50% *(13)*. Currently the recommendation is under review, the discussion is focused on the percentage of the number-based size distribution below the 100 nm to classify a material as nanomaterial. Yet no internationally agreed method to determine the number-based size distribution has been proposed. Therefore we used a combination of analytical methods as suggested by Linsinger *(14)*.

In this study we aimed to provide more information on the number-based size distribution of food grade TiO_2 ingredients and its occurrence in consumer products since this is required to increase the reliability of the human food exposure studies as part of the risk assessment. We used a combination of methods for this, asymmetric flow field-flow fractionation on-line with inductively coupled plasma mass spectrometry (AF4-ICP-MS) and two methods that provide number-based size distributions: scanning electron microscopy (SEM), and single particle ICP-MS (sp-ICP-MS). Comparable size-distributions were found indicating that these methods can reliably be used to enforce food labelling in line with recommendation of the EU definition of nanomaterials.

Experimental

Samples. In this study three types of samples were involved, firstly, several types of TiO_2 food additive E171, secondly food products as cakes, candy and chewing gum, and lastly personal care products as toothpaste. In total seven E171 materials, all food grades and four labelled as E171, were received from suppliers in China, Germany, Italy, the Netherlands and the United Kingdom, and consisted of fine white powders. In total 24 food products and 3 personal care products were investigated. All these products were purchased in 2012 from regular shops in the Netherlands. Since food labelling for titanium dioxide may be imprecise, 20 products that listed "E171", “TiO_2” or "titanium dioxide" on the package were selected, as well as seven products without this labelling but with a typical white colour. Samples were stored in a clean, dry and dark location and analysed before the expiration data.

Determination of total titanium content. For the determination of the total titanium content of the TiO_2 materials a suspension of these materials was prepared according to Jensen *(15)*. In short, ca. 15 mg bovine serum albumine (BSA) is dissolved and diluted in Milli-Q water to reach a final concentration of 10 mg/ml. This solution is filtered over a 0.2µm-pore size filter and diluted twentyfold with Milli-Q water to a final concentration of 0.5 mg/ml BSA. An accurately weighed sample of ca. 15 mg finely powdered TiO_2 material is brought into a 30 ml vial. Then 30 µL of 96% ethanol is added and distributed equally over the TiO_2 material followed by 970 µL of the 0.5 mg/mL BSA solution. The mixture is shaken manually and another 5 ml of the 0.5 mg/mL BSA solution is added and the final suspension shaken manually to obtain a homogenous suspension. While the sample vial is cooled in ice water, the suspension is sonicated with a Misonix XL-2000 sonicator with a CML-4 needle probe for 16 min at 22.5 KHz and 4 W power. The final suspension is used as such, or after further dilution to the desired concentration with the 0.5 mg/mL BSA solution. Suspensions prepared in this way showed to be stable for at least three days. From each suspension a subsample was collected in a perfluoroalkoxy (PFA) digestion vial to which 8 mL of nitric acid (70% HNO_3) and 2 mL of hydrogen fluoride (50% HF) was added. For the determination of the total titanium content of the food and personal care products the whole sample was cut into small pieces, grinded and a representative subsample was used for analysis. An analytical sample of ca. 0.5 g was collected from each sample and brought into a PFA digestion vial to which 6 mL of nitric acid (70% HNO_3), 2 mL of hydrofluoric acid (50% HF) and 2 mL of hydrogen peroxide (30% H_2O_2) was added. All samples were digested in a MARS microwave system for 50 min. The temperature program was as follows; at 1200 W power from 20°C to 150°C in 15 min, then to 180°C in 15 min, and finally constant at 180°C for 20 min. Following digestion and cooling to room temperature, Milli-Q water was added to a total volume of 50 ml. The extracts were shaken manually, diluted to 100 ml and analysed with a Thermo X Series-2 ICP-MS equipped with an autosampler, a conical glass concentric nebulizer, and operated at an RF power of 1400 W. Data acquisition was performed in the selected ion monitoring mode at m/z ratios of 48 and 49 that are characteristic for titanium. Quantification was based on ionic titanium standard diluted in the same acidic matrix. The recovery of the total-Ti method was determined by spiking three products of different matrices in triplicate with known amounts of a TiO_2 reference material NM-103.

Determination of the size distribution in TiO_2 materials using electron microscopy. To determine the size distribution of the titanium oxide nanoparticles in the TiO_2 materials, suspensions of these materials were studied using electron microscopy. TiO_2 suspensions (2.5 mg/ml) were filtered over an Anopore aluminium oxide filter with pore size of 20 nm. The filters were mounted on aluminium specimen holders with double-sided adhesive carbon tape and were coated with a 5 nm layer of chromium using an Emitech K575X turbo sputter coater. The filters were analysed with a high-resolution field emission gun scanning electron microscopy in combination with X-ray analysis (FEG-SEM/EDX). The microscope is a Tescan MIRA-LMH FEG-SEM at an accelerating voltage of 15 kV, working distance 10 mm, spot size 5 nm. The EDX spectrometer is a Broker AXS spectrometer with a Quantax 800 workstation and an XFlash 4010 detector. The SEM is equipped with Scandium SIS software package (Olympus Soft Imaging Solutions, Germany) for automated particle analysis. With this system the filter area is automatically inspected on a field-by-field basis. In each field of view particles are recognized using a pre-selected grayscale video threshold (detection threshold level) to discriminate between particles and filter background. The analysis is conducted using the backscattered electron (BE) mode. From each particle/cluster of particles the projected area equivalent diameter (dpa) is measured. Magnifications were chosen so that their measurable size ranges overlap slightly and cover the particle sizes of interest. To measure dpa values of 25 to 1600 nm, three magnifications of 10.000x, 25.000x and 75.000x were selected. These three magnifications cover eight size bins: 25-40, 40-65, 65-100, 100-160, 160-250, 250-400, 400-650, 650-1000 and 1000-1600 nm. Per size bin a minimum of 10 particles were measured and in total approximately 1000 particles were measured per sample. In order to ensure that there is no overlap of particles, the total loading of the filter with particles was always less than 10%. Per size bin the mean diameter and the 95% confidence interval is calculated on the basis of the Poisson distribution.

Quantification and size determination of TiO_2 particles using AF4-ICP-MS. For quantification and size determination of particulate TiO_2 in TiO_2 materials a suspension of these materials was prepared as described before. For quantification and size determination of particulate TiO_2 in food and personal care products a representative subsample was prepared and 0.5 g of this sample was brought into a 50 ml glass beaker together with 20 ml of hydrogen peroxide (30% H_2O_2). The content of the beaker was heated to about 100°C and kept just below the boiling point of the H_2O_2 matrix. When most of the organic matrix was consumed the content is evaporated until a residue of about 1 ml remains. The content is allowed to cool to room temperature and is diluted to 10 ml with the 0.5 mg/mL BSA solution. Prepared suspensions and extracts are analysed with asymmetric flow field-flow fractionation (AF4) on-line coupled with ICP-MS. The AF4 consisted of a metal free Wyatt Eclipse Dualtec separation system equipped with a 153 x 22 mm flow cell containing a Nadir regenerated cellulose separation membrane with 10 kDa molecular weight cut-off and a spacer of 350 µm. The AF4 system was further coupled to an Agilent 1100 system with an auto-injector and a binary pump for eluent flow delivery and degassing. Prior to injection, samples were thoroughly mixed to ensure homogenous distribution of suspended particles. The injection volume was 10 µl and a 7 min focusing time was used. A constant cross flow of 0.1 ml/min was applied and the flow rate to the detector was kept at 0.5 ml/min. After 57 min the cross flow was reduced to 0 ml/min within 5 min and kept at 0 mL/min for 5 min to remove any residual particles from the separation channel. The outlet flow of the AF4 system passed through a UV detector and was directly coupled with the ICP-MS system for the selective determination of titanium. The ICP-MS system and settings were as described before. Since no well characterised titanium oxide particles were available for calibration purposes, particle size calculation was based on FFF theory using the Boltzmann constant, the column temperature, the eluent viscosity and the instrumental settings *(16, 17)*. In short, the AF4 separation channel consists of an impermeable top block and a bottom block holding a semipermeable ultrafiltration membrane on top of a porous frit. The perpendicular field is caused by restricting the channel flow at the outlet. This restriction will force part of the carrier liquid to leave the channel through the bottom block and thus causing the cross flow. Prior to elution the analyte is concentrated at a position close to the injection port during a focussing step. Particle equilibrium heights above the membrane in AF4 depend on the diffusion coefficient of the particles and the applied cross flow rate with larger particles being driven closer to the membrane. Retention times are dependent on the equilibrium height at which the particles travel in the parabolic flow profile of the channel flow and are be expressed by:

$$t_r = \frac{\pi \cdot \eta \cdot d \cdot \omega^2}{2 \cdot kT} \times \frac{V_{cross}}{V_{channel}} \qquad \text{equation 1}$$

where t_r is the retention time of the analyte; η the viscosity of the eluent; d the hydrodynamic diameter of the particle; ω the channel thickness; k the Boltzmann constant; T the absolute temperature; V_{cross} the cross flow rate and $V_{channel}$ the channel flow rate. With this equation the particle's hydrodynamic diameter can be calculated directly from its retention time. This relation was confirmed by calibration with polystyrene particles. Mass calibration of the ICP-MS was performed with one of the TiO_2 materials for which it was established that the TiO_2 content of this material was close to 100%.

Transformation of mass-based to number-based size distributions. The signal measured by ICP-MS in standard mode is a mass-based signal and the size distribution determined with AF4-ICP-MS is thus also a mass-based distribution. The conversion of a mass- to particle number-based distribution is carried out by placing the detected mass in size bins and calculating the number of particles as follows:

$$N = \frac{M}{\rho \frac{4}{3} \pi (r \times 10^{-7})^3 \times 10^9} \qquad \text{equation 2}$$

Where N is the number of particles, M is the mass detected in a certain particle size bin in ng, ρ is the density of the particle material in g/cm^3 (4.23 g/cm^3 was used for TiO_2) and r is the radius of the particle in that size bin in nm. The numbers are conversion factors to get the units right.

Size determination of TiO_2 particles using single particle ICP-MS. Single particle ICP-MS is a relatively new approach for detection and characterization of nanoparticles and the principle has been described previously *(18,19, 20, 21)*. The ICP-MS instrument and settings used were as described before only in this case data acquisition was done in the time resolved analysis (TRA) mode. The dwell time was set at 3 ms with a typical acquisition times of 60 s per measurement. Because of the short dwell time it is not possible to switch between different isotopes (switching itself takes ~50 ms) and therefore only one titanium isotope, m/z 48, is monitored during measurement. Note that polyatomic interferences are possible, e.g. $^{32}S^{16}O$ and $^{36}Ar^{12}C$, and also isobaric interference by ^{48}Ca. However, these interferences result in a continues background while TiO_2 particles will result in discontinuous signals (peaks) and can thus be distinguished from the background. Since only a part of the nebulized droplets reaches the plasma, the nebulization efficiency needs to be known to determine the actual particle number concentration in the extract. The nebulization efficiency is determined by the analyses of a NIST material, SRM8013, a suspension of 60 nm gold nanoparticles in a citrate buffer, under the same instrumental conditions as the samples but monitoring m/z 197 for gold. The nebulization efficiency is calculated from the observed number of particles in the time scan and the particle flux into the ICP-MS system using equation 3. Single particle data are transferred to and processed in Microsoft Excel for the calculation of particle sizes, particle size distributions and particle concentrations. Acquiring data for 60 s at a dwell time of 3 ms results in 20,000 data points consisting of background signals and signals with a much higher intensity originating from particles (figure 1). These particle signals are isolated from the background by plotting a signal distribution, i.e. the frequency with which a signal height occurs as a function of that signal height. This allows the determination of a cut-off point to separate particle signals from the background (figure 2). From the number of the particle signals in the time scan and the nebulization efficiency determined previously with the gold nanoparticles, the particle number concentration in the diluted sample suspension is calculated as follows:

$$C_p = \frac{N_p}{\eta_n} \times \frac{1000}{V} \qquad \text{equation 3}$$

Where C_p = particle number concentration (L^{-1}); N_p = number of particles detected in the time scan (min^{-1}); η_n = nebulization efficiency; V = sample input flow (mL/min). The same formula is used to calculate the nebulization efficiency after measuring the 50 ng/L 60 nm gold nanoparticle suspension. In that case the particle number concentration C_p is known (50 ng/L of a 60 nm gold particle results in 2×10^7 particles/L) and the nebulization efficiency is calculated from the observed number of particles N_p in the time scan. From the intensity of the particle signals and the response factor calculated from a series of ionic titanium calibration standards that are analysed under the same conditions, the mass of the individual particles is calculated as follows:

$$m_p = \frac{I_p\, t_d}{RF_{ion}} \times \frac{V\, \eta_n}{60} \qquad \text{equation 4}$$

Where m_p = particle mass (ng); I_p = particle signal intensity in the sample (cps); RF_{ion} = ICP-MS response factor from the calibration curve of the ionic titanium standards (cps/µg/L); t_d = dwell time (s); V = sample flow (mL/min). To calculate the particle mass concentration in the diluted sample suspension, the masses of all individual particles are summed and corrected for nebulization efficiency and sample flow:

$$C_m = \frac{\sum m_p}{\eta_n \times V \times 1000} \qquad \text{equation 5}$$

Where C_m = particle mass concentration (ng/L). Finally, the particle size, expressed as the particle's diameter (and assuming a spherical particle shape) is calculated for each particle as follows:

$$d_p = \sqrt[3]{\frac{6\ m_p}{\pi\ \rho_p}} \times 10^4 \qquad \text{equation 6}$$

Where: d_p = particle diameter in the sample (nm) and ρ_p = particle density (g/mL). The individual particle sizes can be used to produce a size distribution graph (figure 3). For all these calculations a template in Microsoft Excel was produced and used throughout.

Results and discussion

We compared the size distributions of TiO_2 particles as determined with three different analytical approaches in three types of samples. Firstly we characterised TiO_2 powders that according to the manufactures are E171 food additives, secondly we characterised food products (cakes, candy, and chewing gum) and finally personal care products (toothpaste).

Titanium content and particle size determination of TiO_2 materials

Total titanium content of TiO_2 materials. The total TiO_2 content of the E171 materials was determined as total titanium using HNO_3/HF digestion and ICP-MS analysis of the extracts followed by recalculation of results to titanium dioxide. The results show that the total TiO_2 content is in the range of 90% to 99% for five of the seven E171 materials and 74% and 83% for the other two materials. Taking into account the measurement uncertainty of the total-Ti method (± 6%) and the fact that these materials may contain some water, five of the seven E171 materials consist of pure TiO_2. It is known that some TiO_2 materials are coated with aluminium- and silicon oxides or polymers to increase photo stability and prevent aggregation *(22, 23)*. Only the packaging of one of the two samples with a lower TiO_2 content declares that the material may contain aluminium hydroxide, amorphous silica, aluminium phosphate and water. No further information about the chemical composition of these two TiO_2 materials was available.

Size distributions of TiO_2 materials determined with SEM. The size distribution of the particles in the TiO_2 materials was determined by SEM. For this the materials were suspended in water and stabilized by BSA *(15)*. Representative SEM images of the TiO_2 materials are shown in figure 4. The images on the left side in figure 4 give a good picture of the large agglomerates that are encountered in the samples while the images on the right side allow the estimation of the primary particle sizes. Detected particles were automatically counted using image analyses software and divided into size bins of 25-40, 40-65, 65-100, 100-160, 160-250, 250-400, 400-650, 650-1000, 1000-1600 nm. The number percentage of the particles in each bin is then plotted against the mean particle size of the size bin to produce a number-based size distribution (figure 4). In general, the size distributions of the seven TiO_2 materials are very alike and range from 30 to 600 nm with the apex in all size distributions between 200 and 400 nm. About 10 % of the particles have dimensions below 100 nm. The actual percentage of particles with at least one dimension smaller than 100 nm will be higher because the image analysis software determines an area equivalent diameter (diameter projected area; dpa) for the particles. As a consequence particles with an aspect ratio different from 1 will be "averaged" into round particles leading to an underestimation of the number of particles with one dimension smaller than 100 nm. From the image analysis it appears that for

>80% of the particles, large and small, the aspect ratio is in the range of 0.8 - 1.2. As a consequence it is expected that the underestimation of the number of particles with one dimension smaller than 100 nm is limited to 10-20%. That all materials appear to be more or less identical probably reflects the production process of this material. The most common production method for TiO_2 particles is the chloride process or the sulphate process *(24)*. In industrialized countries the chloride processes appears to be favoured over sulphate processes for environmental, economic and qualitative reasons *(25)*. Therefore, there is a reasonable change that all investigated TiO_2 materials are produced by the same process.

The smallest size bin used in our measurements was 25-40 nm. Particles in that size range could still be detected, identified and counted by the image analysis software. TiO_2 particles <20 nm cannot be reliably identified and counted by the image analysis software. Manually, the operator can detect TiO_2 particles with diameters down to 10-20 nm in clean suspensions. However, manual detecting small particles is not a reasonable option since many fields have to be viewed in order to produce a reliable particle number. Thus our measurements show that the lower size limit of detection for routine SEM analysis is around 20 nm for TiO_2 particles.

Size distributions of TiO_2 materials determined with AF4-ICP-MS. Strictly speaking AF4 is a separation technique and is not able to determine size, nor a number-based size distribution. As there are no well characterized TiO_2 particles of different sizes (i.e. reference materials for size calibration) we used a theoretical approach based on the well-established theory of flow field-flow fractionation for size calibration as explained in the methods section. The result of the AF4-ICP-MS analysis is a chromatogram in which the signal height is a measure for the mass of titanium detected at a certain retention time. Following mass calibration this signal height is expressed as the mass of Ti or TiO_2 and with the time scale recalculated as a particle size scale based on an equation from flow field-flow fractionation theory described in the experimental section, this results in a mass-based size distribution. However, to apply the EU definition for nanomaterials this mass-based distribution has to be transformed into a number-based distribution. Therefore, this mass-based size distribution is transformed into a number-based size distribution using the equation in the experimental section and assuming that particles have a spherical shape. However, if the mass is converted into a number of particles, this number depends on particle size. If the mass remains constant, as shown in figure 5, the number of particles increases strongly when going to smaller particle (notice that the y-scale is a log scale), especially at small particle sizes, since particle size and mass are related by d^3 (see equation 6 in methods section). This means that mass to number transformations have large conversion factors for small particles that easily lead to erroneous results. In practice, the beginning of chromatograms tends show small spikes, noise, non-particle peaks or small electrical offsets. If these phenomena are also transformed from mass- to number-based, small particle sizes will completely and erroneously dominate the number-based distribution resulting in a dramatic shift of the size distribution to smaller particle sizes. From the chromatograms in this study it was concluded that the smallest TiO_2 particle size that can be determined and reliably transformed from mass- to number-based distributions is around 20 nm.

Comparing the measured size distributions of TiO_2 materials. Conversion of the retention time into particle size and the mass-based distribution into a number-based distribution allows us to derive number-based size distributions for the TiO_2 materials. The AF4 size distribution of the materials for which the EM size distribution was shown in figure 4, are shown in figure 6. In general, the number-based size distribution as determined by EM and AF4 show two types of differences. Firstly, the apex of the size distributions originating from AF4 generally is between 200 and 300 nm while in EM size distributions it is between 200 and 400 nm.

Secondly, the EM size distributions contain larger particles (diameter >600 nm) than the AF4 size distributions. In AF4 large particles (diameter >400 nm) may actually drop on, and roll over, the membrane surface at velocities that are higher than normal elution *(26)*. As a result these large particles elute at shorter retention times thereby simulating to be smaller sized particles. In the number-based size distribution this will become visible as a shift to smaller particle sizes. As before, the percentage of particles with a size <100 nm is determined. In the AF4 analysis this ranges from 10 to 15% with an average of 12%. This is comparable with the 10% of particles with sizes below 100 nm that were found in the EM analyses.

Titanium content and size distributions in food and personal care products

In addition to the TiO_2 materials, food- and personal care products were also analysed for total titanium content and with AF4-ICP-MS for titanium content as well as size distribution. An attempt was made to determine the size distribution of the TiO_2 particles in the extracts of these products with SEM. However, matrix constituents that were still present made it impossible to make a proper image analysis of the particles sizes. As an alternative the extracts were analysed using single particle ICP-MS (sp-ICPMS) since this techniques also produces a number based size distribution. In total 24 food products and 3 personal care products were purchased in regular shops in the Netherlands. Products that listed "E171" or "titanium dioxide" on the package, as well as a few products with a typical "white colour" were selected for this study.

Total titanium content of food and personal care products. All 27 products were digested with a combination of HNO_3, HF and H_2O_2 and the total-Ti content was determined using ICP-MS. Total-Ti recovery was determined by spiking three products of different matrices in triplicate with known amounts of a TiO_2 reference material NM-103. The total-Ti recovery ranged from 92% to 109% with an average of 96 ± 6%. Method blanks showed only low amounts of titanium, i.e. on the average <2 μg of Ti which corresponds to <0.005 mg Ti/g product. The quantification limit of the digestion and detection method was set to 0.01 mg Ti/g product. The total-Ti content of the products ranges from 5.4 mg Ti/g product for a chewing gum to 0.01 mg Ti/g for a white-topped cookie. Four products had total-Ti levels that were below the quantification limit of the method. The highest levels of total-Ti are found in chewing gums and in toothpastes, the lowest in bakery products like cookies and cup-cakes (figure 7). The results are comparable to the total-Ti content in food items reported recently by Weir et al. *(8)*.

Titanium content of food and personal care products determined by AF4-ICP-MS. All food and consumer products with a detectable amount of total-Ti have been analysed with AF4-ICP-MS for particle-based Ti content as well as the particle size distribution. For this purpose a separate sample preparation was set up using H_2O_2 to oxidize the sample matrix. The use of strong acids was avoided since these are not compatible with the AF4 analysis. H_2O_2 oxidation worked for all samples with the exception of the chewing gums were debris of the guar gum remained. Since the TiO_2 particles are expected to be present in the outer, water-soluble coating of the chewing gum *(8)*, the removal of this debris is probably not of influence on the result. The time between sample preparation and measurement with AF4-ICP-MS was kept as short as possible and was no more than 4 hours. After sonication of the sample extract, the extract was kept in constant movement on a roller bank until analysis. Prior to injection the sample was thoroughly mixed to ensure homogeneous distribution of suspended particles. Since ions are removed in AF4 separation during the focusing of the analyte on the semipermeable membrane, mass calibration can only be done with a particle-based standard. In the absence of a reliable, well-characterised TiO_2 particle standard, one of the TiO_2

powders characterized as pure, 99% TiO_2 was used for this purpose. The particle-based Ti content of the food and personal care products as determined with AF4 and expressed as mg Ti particle/g product is presented as the red, right-side bars in figure 7. The side by side comparison of the total-Ti content and the particle-based Ti content as determined with AF4-ICP-MS in each product, shows that they are comparable, certainly at Ti concentrations >0.5 mg Ti/g product. At lower concentration the results from the AF4 determination are generally lower than those from the total ICP-MS method. This can be explained by adsorption processes in the AF4 separation that become visible in the lower concentration range. Another reason may be that very large particles, aggregates or agglomerates (>1600 nm) may be present in the samples and are lost in the AF4 determination. For products containing >0.5 mg Ti/g the results show that most of the total-Ti originates from particles, i.e. TiO_2 particles, and that no large amounts of titanium are unaccounted for in the AF4 analysis.

Number-based size distributions in food and personal care products. Next to the particle-based Ti content of the samples, number-based size distributions were determined (figure 8). From products containing less than 0.1 mg Ti/g product no reliable number-based size distributions could be produced. In general, the calculated number-based size distributions in food items and personal care products based on AF4-ICP-MS analysis are comparable with the number-based size distribution as determined for TiO_2 in E171 materials with EM. The apex of the size distribution was mostly found around 200 nm which is in the size range of the primary particle sizes found with EM. The results indicate that a part of the TiO_2 particles have diameters below 100 nm. On a number-based basis the average percentage of particles with diameters smaller than 100 nm is around 8%, comparable with the 12% and 10% that were found for the number-based size distributions of TiO_2 in E171 with AF4-ICPMS and EM analyses.

Size distributions determined using single-particle ICPMS. Since EM analysis was not feasible in products, single particle ICP-MS (sp-ICP-MS) was used to determine a number-based size distribution of the TiO_2 particles in food and personal care products. The sample extracts that were used for the AF4 analysis were diluted 10,000 times to get in the proper working range of the sp-ICP-MS method. Counting between 300 and 3000 particle peaks in each time scan gives enough information to produce a reliable number-based size distribution. While TiO_2 particles produce discontinuous signals (peaks) in sp-ICP-MS, small particle peaks will disappear in the background noise and can no longer be distinguished. Ti is measured at m/z 48 or 49, which is not that specific or sensitive, respectively. The presence of bi-atomic ions like $^{32}S^{16}O$ and $^{36}Ar^{12}C$, and isobaric interference by ^{48}Ca results in a continues background. This background noise for Ti measured at m/z 48 is substantially higher than for silver (m/z 107) or gold (m/z 197) and therefore the minimum particle size that can be determined for titanium is also higher. While for gold and silver 20 nm nanoparticles can be determined, the particle size detection limit for TiO_2 particles in the sp-ICP-MS method is 50 nm. The use of a collision cell did not improve the situation because not only the background noise was reduced but also the Ti particle peaks resulting in no improvement of the signal/noise ratio.

Comparing the measured size distributions of TiO_2 materials. Compared to the calculated AF4-ICP-MS number-based size distribution (figure 8) the measured sp-ICP-MS number-based size distributions (figure 9) generally are shifted to the left, i.e. toward smaller particle sizes, as illustrated by the results for chewing gum 623 in figure 9. Nevertheless, the AF4-ICP-MS and sp-ICP-MS number-based size distributions of TiO_2 particles in the products are comparable. Furthermore, the distributions of the TiO_2 particles in the products are also comparable with the number-based size distributions of the TiO_2 (E171) materials as determined with AF4-ICP-MS and with EM. In all cases the apex of the size distribution is found around 200 nm within the size range of the primary particle sizes found with EM. The sp-ICP-MS results indicate that 5-10% of the TiO_2 particles have diameters below 100 nm. This is comparable, or somewhat lower than what was found in the EM and AF4-ICP-MS analysis, and may in part be explained by the difference in the smallest particle that can be detected. For EM in the analysis of the TiO_2 materials the smallest TiO_2 particle that could be detected was around 20 nm, for AF4-ICP-MS in the analysis of TiO_2 materials and food and personal care products it was also around 20 nm, while in the sp-ICP-MS analysis of the food and personal care products the smallest detectable TiO_2 particle had a diameter of 50 nm. These practical size detection limits for TiO_2 particles introduce a certain and maybe significant bias due to the that the size range between 1 and 20 nm is excluded. While figure 7 shows that >95% of the total titanium (mass-based) concentration is explained by TiO_2 particles >20 nm, if even only 1% of the total titanium (mass-based) concentration exists of TiO_2 particles <20 nm, this will change the determined number-based particle size distribution significantly. As a consequence it is not clear whether TiO_2 in food products is a nanomaterial and the results especially show the inability of current state of the art methods to support the EU recommendation for the definition of nanomaterials.

Potential for consumer exposure assessment. In this study seven TiO_2 materials and 24 food products and 3 personal care products were studied for their titanium dioxide content and the number-based size distribution of TiO_2 particles therein. Three principally different methods have been used to determine the number-based size distribution of TiO_2 particles in E171 materials and food and personal care products. Comparable size distributions were found and from these it was determined that 5-12 % of the TiO_2 particles in these materials and products were <100 nm. These data are suitable to be used in an exposure study, further refining the exposure assessment by Weir et al for the US population *(8)*. Previously we assessed the SiO2 content of food products *(27)* and subsequently assessed the fate during in vitro digestion *(28)*. There we concluded that the intestinal epithelium is exposed to nano-scale material. The fate of TiO_2 particles during in the human digestive tract is unknown and should be evaluated to allow a comparison with the existing oral toxicity studies used different forms of the TiO_2 NPs. While the applied analytical methods showed practical size limits, all these methods, and especially sp-ICP-MS, can be elegantly employed for further studies on the fate of food grade TiO_2 to bridge the gap between our findings and the recent oral toxicity studies to guarantee the consumer safety.

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Figure 1. Single-particle ICPMS time scan of a diluted sample extracts. Each peak represents one particle. The number of peaks is proportional to the particle concentration is the sample while the peak height is proportional to the particles diameter to the third power.

Figure 2. Signal distribution graph plotting the frequency of the ICP-MS response in data points as a function of the ICP-MS response. ICP-MS responses left from the minimum depict background and ions, those right from the minimum particles.

Figure 3. Typical example of a particle size distribution as produced by single-particle ICP-MS analysis.

Figure 4. Representative SEM images of TiO_2 materials. Image on the left shows the agglomerates while the image on the right shows the individual particles. The number based size distributions produced by image analysis of the SEM images are shown below the SEM images (the error bars indicate the 95% confidence intervals of the number percentage in each particular size bin).

Figure 5. When the mass remains constant the number of particles rapidly increases, especially at smaller particle sizes.

Figure 6. Number-based size distribution of two TiO_2 materials as determined with AF4-ICP-MS and after recalculation from mass-based to number-based.

Figure 7. Total-Ti content in food and personal care products determined with ICP-MS (blue, left-side bars) and the particle-based Ti content in the same products determined with AF4-

ICP-MS analysis (red, right-side bars). The numbers behind the product description refer to sample codes.

Figure 8. Number-based size distribution of the TiO_2 material in a toothpaste and chewing gum sample as determined with AF4-ICP-MS and after transformation of the mass-based to a number-based distribution.

Figure 9. Number-based size distribution of the TiO_2 material in toothpaste as determined directly with sp-ICPMS.

Figure 1

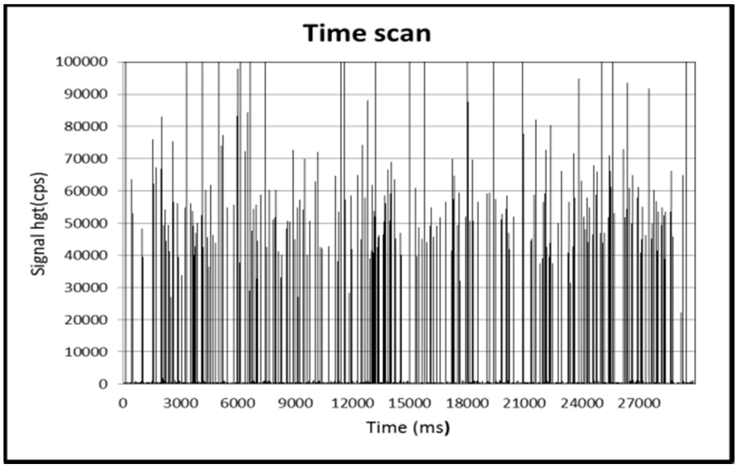


Figure 2

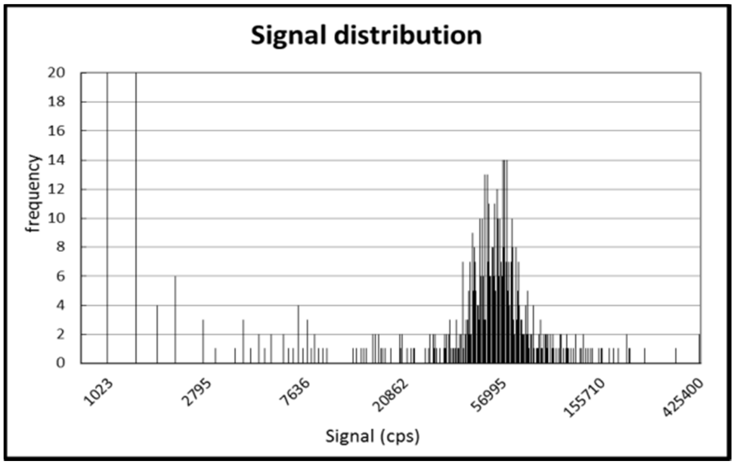


Figure 3

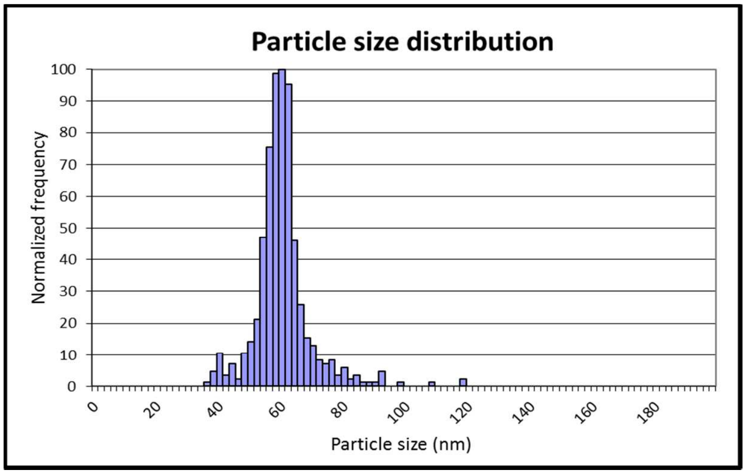
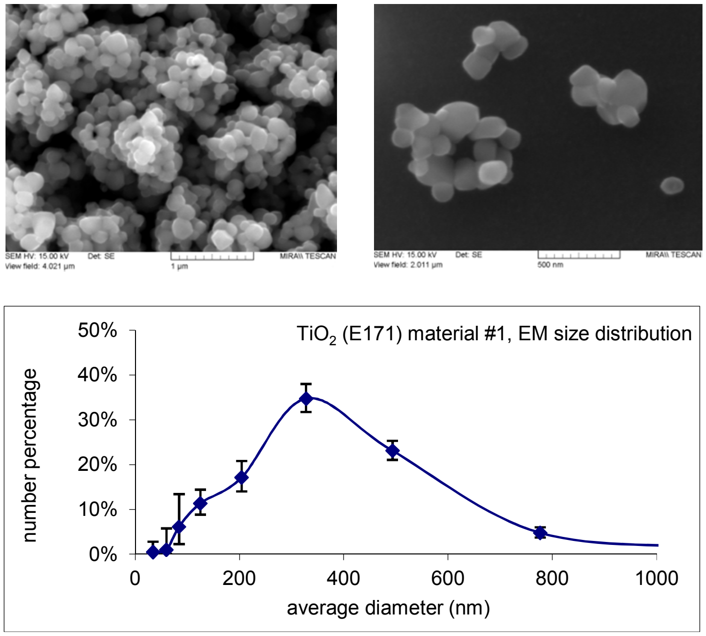
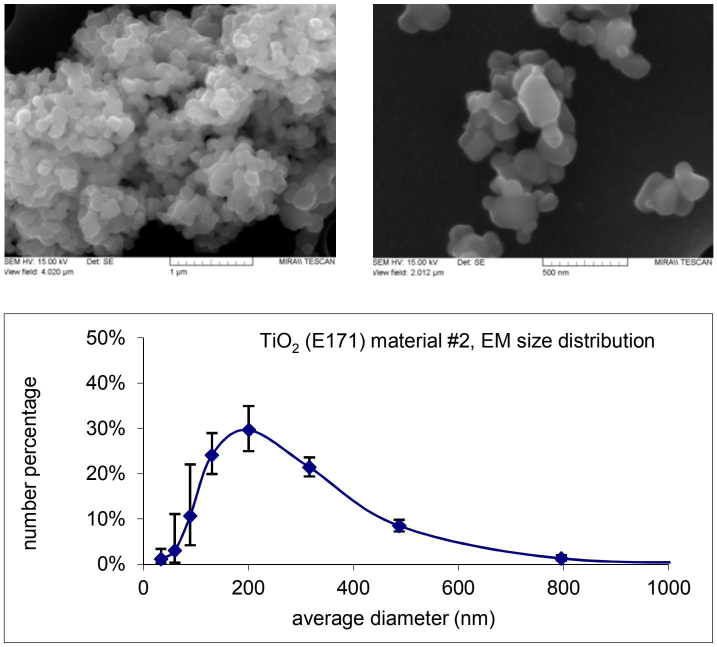

Particle size distribution
Normalized frequency
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
160
180
Particle size (nm)

Figure 4


SEM HV: 15.00 kV
Det: SE
View field: 4.021 μm
1 μm
MIRA\\ TESCAN
SEM HV: 15.00 kV
Det: SE
View field: 2.011 μm
500 nm
MIRA\\ TESCAN
TiO$_2$ (E171) material #1, EM size distribution
number percentage
50%
40%
30%
20%
10%
0%
0
200
400
600
800
1000
average diameter (nm)


SEM HV: 15.00 kV
Det: SE
View field: 4.020 μm
1 μm
MIRA\\ TESCAN
SEM HV: 15.00 kV
Det: SE
View field: 2.012 μm
500 nm
MIRA\\ TESCAN
TiO$_2$ (E171) material #2, EM size distribution
number percentage
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40%
30%
20%
10%
0%
0
200
400
600
800
1000
average diameter (nm)

Figure 5

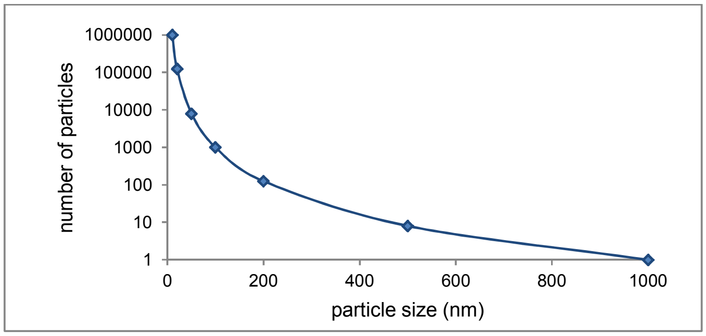


Figure 6

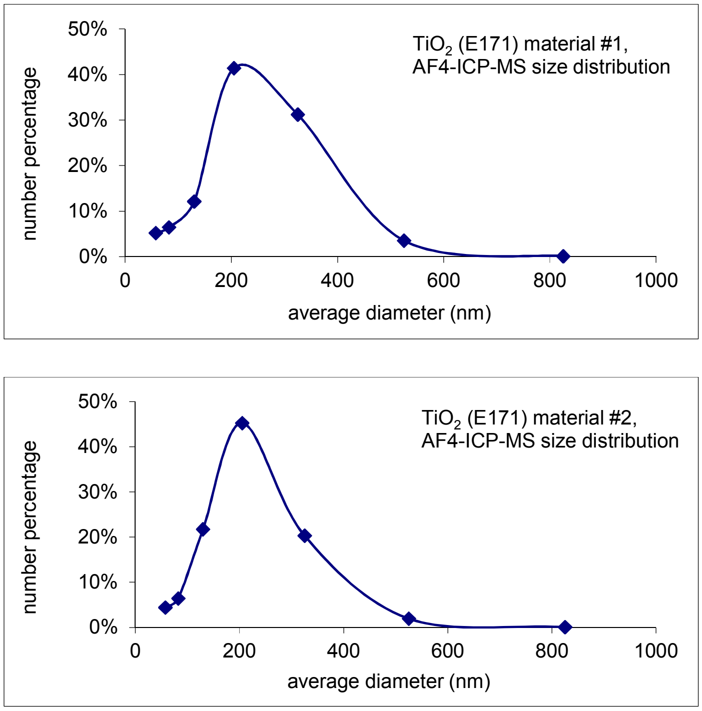


Figure 7

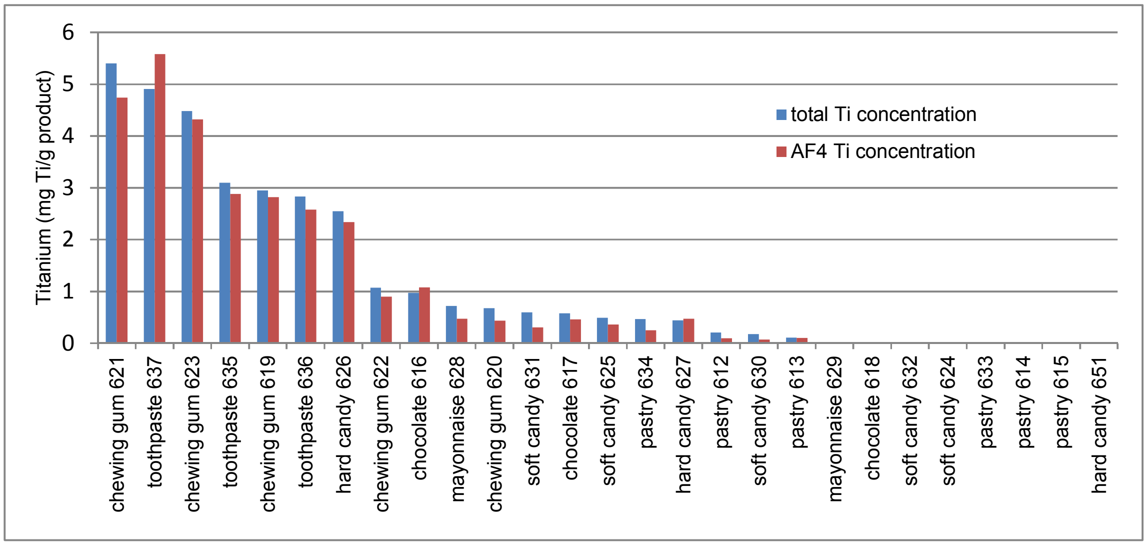
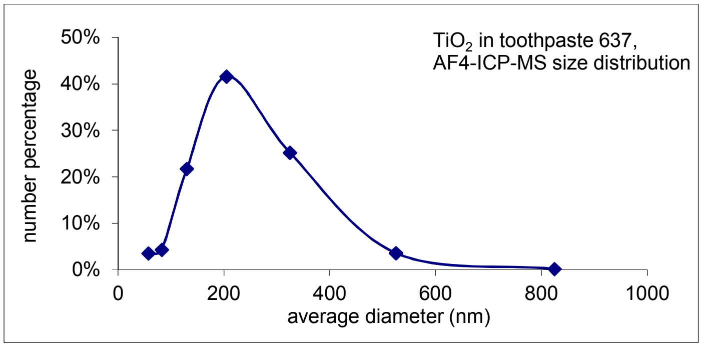


Figure 8



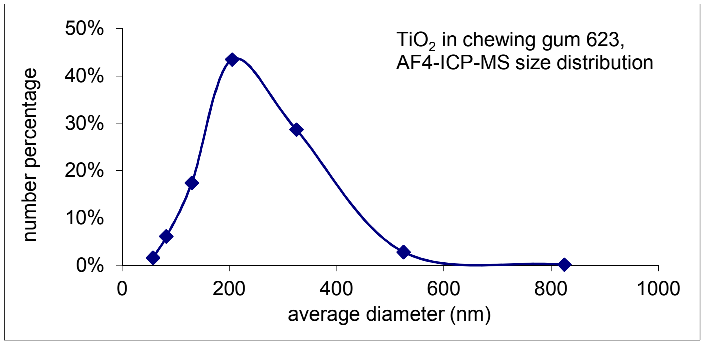
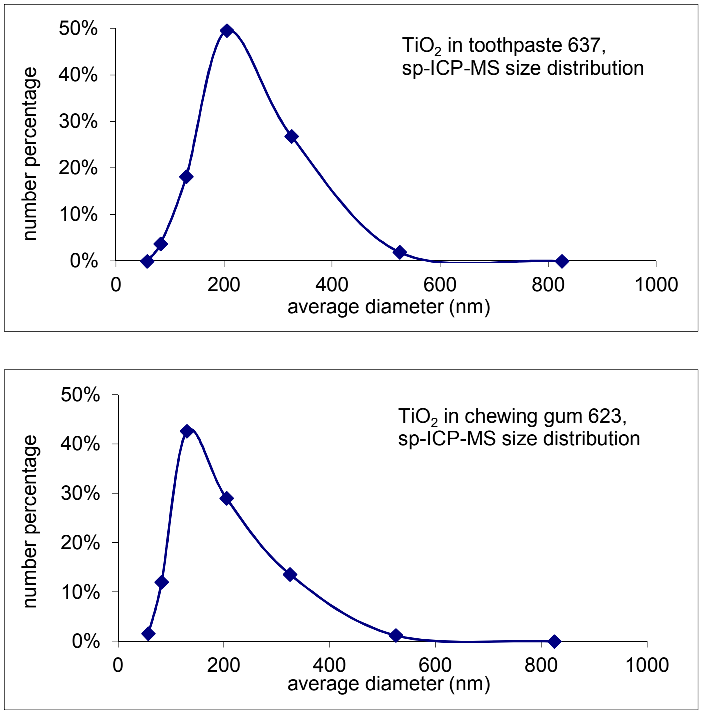
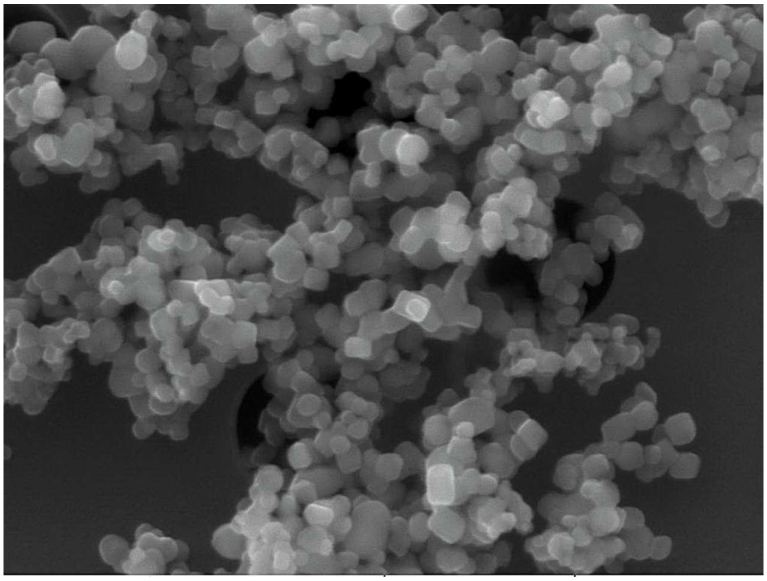


Figure 9


TiO2 in toothpaste 637,
sp-ICP-MS size distribution
number percentage
50%
40%
30%
20%
10%
0%
0
200
400
600
800
1000
average diameter (nm)
TiO2 in chewing gum 623,
sp-ICP-MS size distribution
number percentage
50%
40%
30%
20%
10%
0%
0
200
400
600
800
1000
average diameter (nm)

TOC Graphic

Remark for the editor: I have included this picture because it represents one of the major applications of TiO_2 *in food items, as a whitener and enhancing brightness of coloured products.*



Remark for the editor: The black-and-white EM picture of E171 is included as an alternative in case the colour photo of the candy is not suitable.