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In vitro gastrointestinal stability and Caco-2 cell cytotoxicity of TiO_2 and SiO_2 (nano)particles from confectionary products

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ABSTRACT

The effect of an *in vitro* gastrointestinal assay on the characteristics of nanoparticles from food additives TiO_2 (E171) and SiO_2 (E551) present in confectionaries was determined by spICP-MS. No significant differences were detected in particle size distribution regardless of the phase of the *in vitro* stage and the confectionary products, with particles present as aggregates/ agglomerates. The percentage of TiO_2 as nanoparticle form was found to be less than 50 % during the *in vitro* gastrointestinal digestion process, whereas SiO_2 nanoparticles could not be detected due to the high LOD in size obtained (142 nm). The bioaccessible fractions of intestinal extracts showed a 5 % content for both particle types, suggesting their limited absorption in the body. MTT cytotoxicity assay with Caco-2 cells exposed to gastrointestinal extracts from confectionery products revealed an average cytotoxic effect of 40 % for all products tested which was attributed to the food matrix components rather than (nano) particles.

1. Introduction

Nanoparticles are gaining increasing significance in food industry, playing diverse roles as additives, flavor enhancers, and components in packaging materials. Ensuring the safety and compliance with regulations of these nanoparticles is of paramount importance to safeguard consumer health (DeLoid et al., 2017; Musial et al., 2020).

Titanium dioxide (TiO_2), commonly known as food additive E171, and silicon dioxide (SiO_2) as food additive E551, have become the focus of extensive research due to their ubiquitous presence in various products, ranging from processed foods to nutritional supplements, where they contribute to enhancing physical and aesthetic properties (Geiss et al., 2020; Kesteren et al., 2014). Their analysis is of growing interest owing to their potential impact on food safety and human health (Baranowska-Wójcik et al., 2022). The use of the food additive E171, widely used as whitening agent, has been prohibited by European authorities (The European Commission, 2022), whereas in other regions like the U.S, its utilization remains authorized. Despite the ban on TiO_2 as a food additive, there is still some uncertainty regarding its direct impact on human health. On another note, the food additive E551, commonly used as anticaking agent, maintains its permissibility despite undergoing multiple reassessments by competent authorities (EFSA, 2018). However, it remains under the discerning scrutiny of researchers by means of conducting toxicity studies on these nanoparticles in humans. The focal point of these studies lies in understanding the fate of these nanoparticles post oral ingestion (Jovanović, 2014). Nevertheless, the available literature lacks *in vitro* assays considering the food matrix in which these nanoparticles are embedded (Cao et al., 2020) and the impact of physicochemical factors occurring along the gastrointestinal tract (variation of pH, presence of carbohydrates, lipids, or proteins) on the characteristics and properties of nanoparticles (DeLoid et al., 2017; McClements et al., 2017; Espada-Bernabé et al., 2024).

Furthermore, the determination of nanoparticles, specifically TiO_2 and SiO_2, has become critically important due to accumulating evidence suggesting their involvement in instigating inflammatory processes at the cellular level within the intestinal epithelium (Tada-Oikawa et al., 2016; Talamini et al., 2019). This has potential implications for the integrity of the intestinal barrier and the homeostasis of the digestive system. Additionally, recent hypothesis suggests that these nanoparticles might exert an impact on the intestinal microbiota, a key factor affecting gastrointestinal health and the equilibrium of the immune system (Chen et al., 2017; Guilloteau et al., 2022; Yan et al., 2022).

To evaluate the mentioned aspects, the development of analytical techniques is essential to enable a comprehensive characterization of nanoparticles in complex food matrices such as confectionery products (Givelet et al., 2021; Laborda et al., 2016). Among these techniques are

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electron microscopy techniques, including Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) coupled with Energy Dispersive X-Ray (EDX), as well as light scattering techniques like Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA), and Multi Angle Light Scattering (MALS) (Brar & Verma, 2011; Verleysen et al., 2019). However, obtaining information on the number and mass concentration with these techniques can be challenging. In last years, spICP-MS (Single Particle Inductively Coupled Plasma Mass Spectrometry) has focused the interest of researchers as it provides relevant information on nanoparticles physicochemical properties (size distribution, number and mass concentration or ionic concentration) (Laborda et al., 2014; Mozhayeva & Engelhard, 2020).

Therefore, the main goal of the current study focuses on investigating the gastrointestinal fate of TiO_2 and SiO_2 (nano)particles found in samples of confectionery products (pure cocoa, soluble cocoa, and powdered custard) by using an *in vitro* gastrointestinal assay and the Caco-2 cell line. The morphology, aggregation/agglomeration state, size distribution and particle number concentration at each stage of the process will be determined by TEM and spICP-MS techniques and percentage of bioaccessibility will be calculated. Moreover, Caco-2 cell line will be exposed to the intestinal fractions, and cell viability will be assessed at various exposure times using the MTT colorimetric assay.

2. Materials and methods

2.1. Samples

In this study, the confectionery products (pure cocoa powder, soluble cocoa powder, and powdered custard) that may contain TiO_2 and/or SiO_2 (nano)particles were subjected to an *in vitro* gastrointestinal digestion model. All these products were acquired from supermarkets in the Community of Madrid, Spain.

Ingredient lists of each confectionery product can be found in Supplementary material.

2.2. Titanium and silicon total content

Total titanium and silicon content in the confectionary products was performed by an Agilent 7700x ICP-MS (Agilent Technologies Inc., USA) instrument, after acid digestion in a microwave oven (MSP 1000, CEM, Matheus, NC). The mineralization protocol was adjusted based on Espada-Bernabé et al., 2024, with a modification to set the sample mass at 100 mg. For both titanium and silicon quantification, an external calibration was established using a dissolved titanium and silicon ionic standard (Merck, Spain) ranging from 0 to 100 μg L^{-1} and 0 to 500 μg L^{-1}, respectively with an acid content equivalent to that of samples resulting from the digestion procedure. Data was collected under continuous acquisition mode by monitoring ^{48}Ti and ^{28}Si isotopes. Detection and quantification limits were calculated as three and ten times the blank standard deviation, respectively (IUPAC, 1978). The experimental parameters were set as follows: RF power at 1550 W, sample depth of 8 mm, plasma gas flow rate of 15.0 L min^{-1}, auxiliary gas flow rate of 0.90 L min^{-1}, and a Micromist nebulizer with a flow rate of 1.01 L min^{-1}. Daily adjustments of ICP-MS parameters, including nebulization gas flow, torch position, optical lenses, and quadrupole voltages, were carried out. Analyses were performed in triplicate.

2.3. In vitro gastrointestinal digestion assay. Study of bioaccesibility of TiO_2 and SiO_2 (nano)particles from confectionery products

An *in vitro* gastrointestinal digestion process implies the application of different centrifugation steps along the process. In a previous study, Espada-Bernabé et al.(2024) showed that centrifugation (speed and time) has a notably effect on TiO_2 particles sedimentation, being the 1500 rpm and 5 min the optimal conditions. Therefore, same centrifugation conditions (5 and 10 min at 1500, 4000, 6000, and 10000 rpm speeds) were evaluated for SiO_2 (200 nm Silica Nanospheres. Nanocomposix, Spain) aqueous particle standard by spICP-MS measurements.

The *in vitro* simulated gastrointestinal digestion experiments were carried out under the same conditions described by Espada-Bernabé et al.(2024), consisting of three distinct phases (namely salivary, gastric and intestinal). Briefly, 1.0000 g of each confectionary product was dissolved in 10 mL of Milli-Q water. In the salivary step, the 10 mL of aqueous extract was combined with 5 mL of *in vitro* synthesized saliva containing α-amylase, agitating the mixture at 200 rpm and maintained at 37 °C for 15 min. Subsequently, 12 mL of gastric solution containing pepsin were added and the solutions were acidified to a pH of 1.8 using HCl and maintained under stirring at 200 rpm at 37 °C for 2 h. The gastric process was stopped by raising the pH to 6.8 using a saturated sodium bicarbonate solution. Finally, 17 mL of simulated intestinal fluid that includes pancreatin were added to simulate the intestinal phase, and the resulting solutions were subsequently incubated at 200 rpm and 37 °C for 2 h. Aliquots of each step were taken for its further characterization by TEM and spICP-MS as changes in (nano)particle size, morphology and aggregation state may occur and by ICP-MS to assess the total content of titanium and silicon in each *in vitro* gastrointestinal stage. The acid digestion mixture and conditions were applied as described in section 2.2. Three replicates were performed for each confectionary product.

Moreover, the bioaccessibility percentage was also calculated from each gastrointestinal stage using Eq. (1):

$$\%Bioaccessibility = \frac{[Ti/Si]_{totalgastrointestinalextract}}{[Ti/Si]_{totalcontent}} \times 100 \quad (1)$$

where $[Ti/Si]_{\text{total gastrointestinal extract}}$ refers to the total titanium or silicon content identified in the salivary, gastric, or intestinal extracts, while $[Ti/Si]_{\text{total content}}$ represents the total titanium or silicon content found in the food products after acid digestion process described in Section 2.2. This formula provides an estimate of the proportion of Ti/Si that is bioaccessible or available for being absorbed after the gastrointestinal digestion process.

2.4. Assessment of changes in the physicochemical properties of TiO_2 and SiO_2 (nano)particles during the in vitro gastrointestinal digestion process

Since the conditions of the different stages of *in vitro* gastrointestinal digestion assay can affect several physicochemical properties of the particles studied, such as size, aggregation/agglomeration state, and morphology; complementary techniques have been employed to assess the change in these properties.

Hence, it is essential to compare the different particle parameters before and after the gastrointestinal digestion process. To determine the physical–chemical characteristics of nanoparticles before performing the *in vitro* gastrointestinal assay, confectionary products were treated with water following the adapted procedure described by Espada-Bernabé et al.(2024). In summary, 100 mg of the sample was suspended in 10.0 mL of Milli-Q water and manually shaken for 10 min to quantitatively extract the (nano)particles. On the other hand, the fractions of the gastrointestinal extracts were obtained as described in Section 2.3.

2.4.1. TEM analysis

The collected fractions from each stage of the *in vitro* gastrointestinal digestion study were characterized in terms of morphology and aggregation/agglomeration state by TEM. Around 25 μL aliquots from each stage of the gastrointestinal process were collected and placed onto copper grids and allowed to air-dry. These dried samples were then subjected to TEM and energy dispersive X-ray spectroscopy (EDX) measurements with a JEOL JEM 1400 PLUS instrument, which operates at 140 kV and is equipped with a CCD camera (KeenView Camera) (JEOL Ltd., Tokyo, Japan). Constituent particle size and distributions from TEM micrographs were obtained according to rule number 4 of Part 3:

Standard operating Procedures (SOPs) from The NanoDefine Methods Manual (Mech et al., 2020).

2.4.2. spICP-MS analysis

TiO_2 and SiO_2 (nano)particles size distribution and concentration (part L^{-1} or part g^{-1}) derived from each *in vitro* gastrointestinal digestion stage were assessed by spICP-MS. An Agilent 7700x quadrupole ICP-MS was employed, featuring a peristaltic pump, a Micromist nebulizer, a cooled Scott-type spray chamber, and a standard quartz torch with a 1.0 mm inner diameter. A dwell time of 3 ms was established, and the overall data acquisition time was set to 60 s. Operational parameters were set daily to achieve peak sensitivity. The transport efficiency was calculated using a diluted 30 nm quality control material of citrate-coated gold nanoparticles (AuNPs) (LGCQC5050) at a concentration of 20 ng L^{-1} and monitoring ^{197}Au signal. Transport efficiency mean value of 3.7 ± 0.2 % was calculated according to the particle frequency method developed by Pace et al.(2011). A mean flow rate of 0.30 ± 0.02 mL min^{-1} was daily gravimetrically calculated as the variation in mass of Milli-Q water introduced into the equipment over a period of 15 min.

To mitigate the interference of particle signals-background overlap, and double events in spICP-MS measurements, substantial dilution factors were employed for the samples, as outlined by Bolea-Fernández et al.(2017). The diluted sample suspensions produced around 600–1200 particles detected during the selected acquisition time. This particle number considers particle concentration ranges between $2{\cdot}10^6$ to $2{\cdot}10^8$ particles L^{-1}, deem enough to ensure a statistically sound particle size distribution under theses experimental conditions according to standard operating procedure proposed by Mech et al. (2020) in The NanoDefine Methods Manual Part 3: Standard operating Procedures (SOPs). In addition, for each stage, data were acquired in triplicate for the ^{48}Ti and ^{28}Si isotopes separately. Calibration curve of ionic Ti and Si ranging from 0 to 75 μg L^{-1} and 0 to 500 μg L^{-1}, respectively, were prepared daily by employing ionic standard solutions for ICP-MS.

Raw signal intensity data were processed using the free RIKILT spreadsheet for Microsoft Excel described by Peters et al.(2015). This facilitated the calculation of particle number concentration and particle mass concentration. For obtaining TiO_2 particle size a TiO_2/Ti ratio of 1.667 and a density of 3.9 g cm^{-3} (anatase phase) were selected. This choice is based on anatase being the predominant crystalline form employed for producing food-grade TiO_2 (EFSA Panel on Food Additives and Flavourings (FAF), 2019). TiO_2 (nano)particles measurements by spICP-MS were validated with a TiO_2 powder refereed as NM-100-JRCNM10200 from the European Commission Joint Research Center (JCR) (Ispra, Italy) being composed mainly of anatase. According to a characterization carried out by JRC using TEM, the TiO_2 particles of NM-100 have a mean diameter of 190.6 ± 5.8 nm (European Commission. JRC, 2014). For SiO_2 particles, a SiO_2/Si ratio of 2.143 and a density of 2.6 g cm^{-3} were set to obtain the same parameters as above. SiO_2 determinations by spICP-MS were validated with 200 nm Silica Nano-Spheres (Nanocomposix, California). For the characterization of both (nano)particles in terms of size (diameter) and concentrations it was assumed that the particles are spherical.

2.5. Effect of intestinal extracts on Caco-2 cells viability by MTT assay

MTT assay allows to evaluate the cytotoxicity of intestinal extracts containing TiO_2 and SiO_2 (nano)particles on Caco-2 cells.

Human Caco-2 cells (ATCC, American Type Culture Collection, U.S.) were cultured in DMEM (Dulbecco's Modified Eagle Medium) (Fisher Scientific, Spain) supplemented with 10 % FBS (Fetal Bovine Serum) and 5 % penicillin–streptomycin. The cells were incubated and maintained in plastic dishes at 37 °C in a humidified atmosphere of 5 % CO_2. Culture medium was refreshed every 2 to 3 days until the cells reached 80 % confluence. Upon this point, the cells were dissociated using PBS (Phosphate-Buffered Saline) (Fisher Scientific, Spain) at pH 7.4 and Trypsin-EDTA (0.05 %) (Fisher Scientific, Spain). Cells were resuspended in DMEM.

To evaluate the potential effect of ionic titanium and silicon, TiO_2 and SiO_2 as (nano)particles and its overall effect on cell viability, Caco-2 cells were exposed to the different chemical forms at concentration found in intestinal extracts after *in vitro* digestion. Consequently, concentrations of 20 and 40 μg L^{-1} for ionic titanium (Merck, Spain), 200 and 1000 μg L^{-1} for ionic silicon (Merk, Spain), 1 and 10 μg L^{-1} (10^6-10^7part g^{-1}) for particulate TiO_2 (Merk, Spain) and SiO_2 (10^6-10^7 part g^{-1}) (200 nm Silica NanoSpheres. NanoComposix, California) were evaluated on cell viability of Caco-2 cells by MTT assay. For that purpose, 15,000 cells per plate (optimized number) were seeded in a 96-well plate and maintained with DMEM at 37 °C in a humidified atmosphere of 5 % CO_2 overnight. Negative controls with only culture medium in absence of cells and positive controls, containing cells in DMEM supplemented with 10 % FBS, were also evaluated. The culture medium was then removed, and the cells were exposed to the commercial solutions, during 4 and 24 h. In the case of positive and negative controls, an equivalent volume of culture medium was added instead of FBS. After each incubation time, the culture media was removed, 20 μL of a 5 mg mL^{-1} MTT solution was added, and cells were then incubated under identical conditions for 4 h. Afterwards, the MTT solution was removed, and the resulting formazan crystals were dissolved in 100 μL of DMSO. Finally, the absorbance was measured at 570 nm using a microplate reader (Fisher Scientific, Hampton, EEUU).

The same protocol was applied on Caco-2 cells exposed to intestinal extracts and their respective blanks (intestinal solution) after *in vitro* digestion, both diluted by a factor of 1/10 in DMEM supplemented with 10 % FBS. The same dilution factor was applied to ionic titanium and silicon and to TiO_2 and SiO_2 as (nano)particle standards.

2.6. Statistical analysis

One- and two-way ANOVA analysis were performed to identify any statistically significant differences during the gastrointestinal process concerning the concentration and constituent particle size of each analyzed confectionery product. The ANOVA analysis was conducted using Statgraphics 18 software.

3. Results and discussion

3.1. Total content of titanium and silicon in confectionary products

The ICP-MS analysis was employed to determine the total silicon and titanium content in the confectionery products chosen for this study. This was conducted after subjecting the samples to an acid mineralization process, aiming to uncover the potential presence of TiO_2 and SiO_2 as these compounds might not always be explicitly listed as food ingredients.

Interferences for most abundant isotopes of both elements, ^{48}Ti (73.73 % of relative isotopic abundance) and ^{28}Si (92.23 %), we carefully considered. In the case of ^{48}Ti, the potential polyatomic interferences from $^{32}S^{16}O$ and $^{36}Ar^{12}C$, and the isobaric interference of ^{48}Ca are well reported in literature (Bucher & Auger, 2019; Peters et al., 2014). For this reason, ^{48}Ca (0.187 %) was also monitored in both the samples and the blanks, with no significant differences found in the signals. It was therefore concluded that the calcium signal was low enough to have no effect on titanium.

Regarding ^{28}Si isotope, there are numerous works in the literature referring to the significant polyatomic interferences, such as $^{12}C^{16}O$ and $^{14}N_2^+$, of this isotope when measured in a single quadrupole ICP-MS (Bolea-Fernández et al., 2017). Trying to minimize these interferences, both ^{28}Si and ^{29}Si isotopes were monitored with and without H_2 as gas cell. No significant improvements in sensitivity were observed, resulting for ^{28}Si LODs of 40 and 39 μg L^{-1} for non-gas and gas mode, respectively.). Hence, ^{28}Si isotope was monitored for subsequent measurements in non-gas mode.

There were no significant differences in LOD and LOQ values for titanium between Milli-Q water and confectionery product extracts, with a LOD of 5 μg L^{-1} and a LOQ of 14 μg L^{-1}. However, silicon showed variations, with Milli-Q water providing a LOD of 40 μg L^{-1} and a LOQ of 121 μg L^{-1}, while confectionery products displayed higher values, with LODs ranging from 74 to 86 μg L^{-1}L and LOQs from 223 to 262 μg L^{-1}.

Matrix effect was also evaluated, as confectionary products are complex samples. The comparison of slopes between standard addition and external calibration revealed no significant differences ($p > 0.05$) for any of the confectionery products.

Both titanium and silicon were also quantified in all examined confectionery products as indicated in Table 1. Comparable levels of titanium were measured in pure cocoa powder, soluble cocoa powder, and powdered custard. By contrast, silicon contents varied significantly among the different products, generally displaying higher concentrations compared to titanium, as expected according of its high abundance in Earth's crust (Silicon is the second most abundant element after oxygen).

3.2. Bioaccesibility of TiO_2 and SiO_2 (nano)particles from confectionary products

To assess the availability of TiO_2 and SiO_2 (nano)particles for absorption in the intestine, the bioaccessibility percentages were calculated for the different confectionery products. The total Ti/Si content was measured in each stage of the gastrointestinal process (Section 2.3), as well as the total content of the products before gastrointestinal simulation (Section 2.2).

According to the data collected in Table 2 the highest bioaccessibility values was obtained for silicon in soluble cocoa, with silicon presenting a higher bioaccesibility than titanium regardless the confectionary product in the *in vitro* intestinal stage.

3.3. Variations in particle concentration, size distribution, constituent particle size, and morphology throughout the gastrointestinal tract

Particles contained in confectionery products can undergo changes in their physicochemical properties along the gastrointestinal tract. These changes can be related to morphology, particle concentration, and size distribution as (nano)particles interact with different biological components employed in the *in vitro* digestion assay. In addition, pH variations at each stage of the gastrointestinal process could play an important role in these changes.

Quantitative information such as size distribution and particle concentration could not be obtained from TEM analysis. In the micrographs shown in Fig. 1 for all confectionaries extracts, aggregated particles of small size were observed. Due to the complexity of the extracts in terms of food matrix, enzymatic composition as well as the low levels of TiO_2 and SiO_2 particles, it was not possible to obtain a significant number of particles to determine particle size. The presence of SiO_2 particles was confirmed by EDX (Kα (1.740 keV)) for all the confectionary products. However, titanium signal was only detected for powdered custard (Kα (4.508 keV) and Lα (0.452 keV)) in salivary stage, which could be attributed to the low level of Ti found in the extracts.

Since no quantitative information could be obtained by TEM, spICP-MS analysis was performed under the conditions previously described in section 2.4.2. The 5σ criterion (σ: standard deviation from baseline) was applied to distinguish between background signals and particulate matter, thus mitigating the occurrence of false positives in the spICP-MS analysis (Laborda, Giménez-Ingalaturre, Bolea, & Castillo, 2020). This approach considers the frequency distribution of recorded events, represented via histograms. Applying this criterion, LOD_{size} of 30 nm for TiO_2 and 142 nm for SiO_2 in Milli-Q water were obtained. The high LOD_{size} value for SiO_2, observed when measurements were conducted using a single quadrupole ICP-MS, hindered the detection and quantification of nanoparticulate material bellow 100 nm.

As for conventional ICP-MS determinations (Section 3.1), the interferences of each isotope analyzed were considered in the spICP-MS measurements. For TiO_2 determinations in non-gas mode, isobaric interference between ^{48}Ti and ^{48}Ca was addressed by monitoring ^{48}Ca in the samples. Low levels of calcium were detected as a continuous background, from which ^{48}Ti could be clearly differentiated. Therefore, the ^{48}Ti isotope was monitored in non-gas mode. Similar conclusions were reported by Bucher & Auger.(2019) when analyzing food products containing E171, suggesting that the mentioned isobaric interference can be disregarded when measuring this isotope in such samples using spICP-MS.

For silicon, the high backgrounds posed a challenge in distinguishing the particles from the ionic background. Similar to conventional ICP-MS determinations, H_2 was tested as a collision gas to achieve a signal background between 0–5 μg L^{-1} (Bolea-Fernández et al., 2017), but no significant improvements in the recorded backgrounds were found.

Due to the lack of TiO_2 reference material, the results were validated by the analysis of NM-100-JRCNM10200, which was extensively characterized in terms of size (European Commission. JRC, 2014). According to JRC, the average diameter of TiO_2 particles in the NM-100-JRCNM10200 material is 190.6 ± 5.8 nm, with a nanoparticulate fraction of 27.1 %. Comparable results, with a constituent particle size of 193 ± 5 nm and a nanoparticulate fraction of 25 ± 2 %, were obtained when measuring this material under the conditions described in section 2.4.2. In addition, the obtained results for this material were comparable to those obtained by Bastardo-Fernández et al.(2023), who performed the measurements by using a spICP-MS/MS with a high efficiency sample introduction system. On the other hand, the validation of the SiO_2 results obtained by spICP-MS, in terms of size, was performed using a 200 nm standard Silica Nanospheres. The constituent particle size obtained by monitoring ^{28}Si was 200 ± 6 nm.

A key factor in evaluating changes in the physicochemical properties of nanoparticles and obtaining consistent results is to avoid nanoparticle sedimentation during the centrifugation steps applying between gastrointestinal digestion phases. Therefore, preliminary centrifugation tests were performed at the specified times and speeds outlined in Section 2.3. The selected optimal centrifugation conditions for both particles were 5 min at a speed of 1500 rpm, resulting in recoveries of 89 ± 6 % for TiO_2 and 93 ± 2 % for SiO_2, obtained by spICP-MS analysis. Once selected the optimal centrifugation conditions, samples were submitted to the *in vitro* gastrointestinal digestion process and the resulting extracts obtained after applying the optimized centrifugation conditions from

Table 1
Total titanium (Ti) and silicon (Si) content in pure and soluble cocoa, and custard powder assessed by ICP-MS.

Food matrix	^{28}Si μg g^{-1}	^{48}Ti μg g^{-1}
Pure cocoa	2172 ± 50	50 ± 1
Soluble cocoa	1315 ± 27	34 ± 1
Powdered custard	387 ± 40	38 ± 1

Results expressed as the mean value ± SD for n = 3 determination.

Table 2
Titanium (Ti) and silicon (Si) percentages from salivary, gastric and intestinal stages. Data obtained by ICP-MS.

In vitro digestion stage	% Titanium			% Silicon		
	Pure cocoa	Powdered custard	Soluble cocoa	Pure cocoa	Powdered custard	Soluble cocoa
Salivary	8 ± 1	10 ± 1	9 ± 2	20 ± 4	5 ± 2	3 ± 1
Gastric	9 ± 1	11 ± 2	40 ± 7	40 ± 8	2 ± 1	3 ± 1
Intestinal	19 ± 3	18 ± 1	45 ± 10	45 ± 9	26 ± 5	78 ± 2

Results expressed as the mean value ± SD for n = 3 determination.

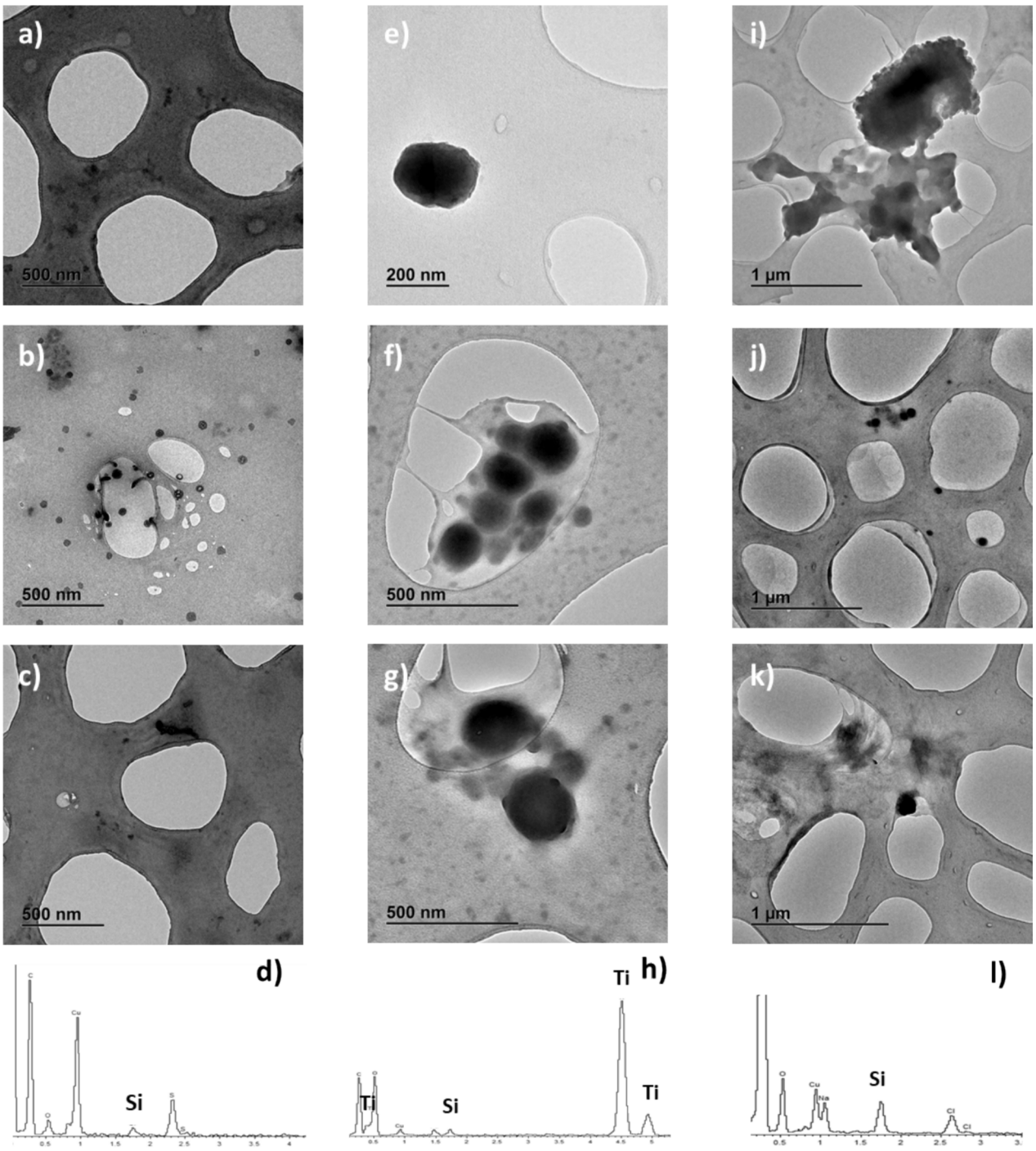

Fig. 1. TEM micrographs of soluble cocoa (a), b) and c)), powdered custard (e), f) and g)), pure cocoa (i), j) and k)) extracts across gastrointestinal simulation stages a), e) and i) salivary, b), f) and j) gastric and c), g) and k) intestinal. Figures d), h) and l) shows EDXs signals from each simulation stage, respectively.

each step were measured by spICP-MS (Section 2.4.2). The analysis of the extracts from the different gastro-intestinal steps allowed us to determine the step with the major incidence on the size and concentration of nanoparticles. It is important to emphasize that the use of enzymes resulted in an increase in the signal background for both elements. The LOD_{size} for TiO_2 increased from 30 to 63, 56, and 61 nm in the salivary, gastric, and intestinal phases, respectively. However, for SiO_2, a significant increase in the signal background was only observed in the intestinal stage, resulting in an increase in the LOD_{size} from 142 nm in water, salivary, and gastric phases to 151 nm in the intestinal phase. In addition, it is important to highlight that no TiO_2 or SiO_2 particles were detected in the salivary, gastric or intestinal enzymatic blanks.

The size variations of TiO_2 and SiO_2 (nano)particles detected in saliva, gastric, and intestinal extracts for each confectionary product studied are shown in Table 3. No significant differences ($p > 0.05$) were found in the constituent particle size and size distribution of TiO_2 particles between different phases of the *in vitro* gastrointestinal digestion of the confectionary products when performing one-way ANOVA analysis. Our results partially agreed with those reported by Ferraris et al.(2023) and Bettencourt et al.(2020) where size distribution of particles derived from bulk food additive E171 remains unaltered during *in vitro* simulation.

Similarly, the size distribution of SiO_2 particles remains unaltered (p

Table 3
TiO_2 and SiO_2 (nano)particle characterization in terms of mean size, percentages under 100, 250 and 500 nm before and after the *in vitro* gastrointestinal digestion steps for pure cocoa, custard powder and soluble cocoa. Measurements were performed by spICP-MS.

		TiO_2 particles			SiO_2 particles		
		Constituent particle size (nm)	<100 nm (%)	<250 nm (%)	Constituent particle size (nm)	<250 nm (%)	<500 nm (%)
Pure cocoa	Water	126 ± 40	30 ± 4	98 ± 3	269 ± 27	32 ± 7*	100
	Salivary	127 ± 37	24 ± 6	98 ± 5	257 ± 61	67 ± 12	100
	Gastric	128 ± 39	25 ± 4	98 ± 5	248 ± 65	64 ± 11	99 ± 2
	Intestinal	134 ± 28	ND	99 ± 6	238 ± 60	69 ± 11	99 ± 3
Powdered custard	Water	147 ± 72	36 ± 7	97 ± 2	278 ± 36	37 ± 9*	98 ± 5
	Salivary	145 ± 41	38 ± 5	98 ± 2	258 ± 60	85 ± 13	99 ± 3
	Gastric	146 ± 34	37 ± 6	97 ± 5	220 ± 47	80 ± 11	99 ± 3
	Intestinal	151 ± 42	ND	97 ± 4	261 ± 54	86 ± 9	98 ± 4
Soluble cocoa	Water	125 ± 65	48 ± 6	97 ± 4	271 ± 21	43 ± 11*	100
	Salivary	156 ± 28	ND	99 ± 3	218 ± 36	82 ± 15	100
	Gastric	149 ± 27	ND	97 ± 5	207 ± 39	87 ± 17	100
	Intestinal	167 ± 40	ND	93 ± 4	235 ± 28	83 ± 10	100

ND: not detected.
* Indicates statistically significant differences ($p < 0.05$) within the same column before (water) and under *in vitro* conditions (salivary, gastric and intestinal).

> 0.05) during the different phases of *in vitro* gastrointestinal digestion process according to one-way ANOVA. In other reported studies the results obtained are contradictories, Peters et al.(2012), indicated the formation of SiO_2 agglomerates during gastric digestion whereas McClements et al.(2016) showed that an increase in pH during the gastric phase could induce the deagglomeration of SiO_2 particles. In our case, only SiO_2 particles larger than 142 nm were detectable and quantifiable because of the LOD_{size} values according to the single quadrupole ICP-MS used in the measurements. As it can be seen in Table 3, in the case of TiO_2, the percentage of TiO_2 as nanoparticle form is less than 50 % during the different phases of the *in vitro* gastrointestinal digestion process. In the case of SiO_2 nanoparticles could not be detected due to the high LOD_{size} (142–151 nm).

The variation in the concentration and (nano)particle percentage of TiO_2 and SiO_2 material throughout the *in vitro* gastrointestinal digestion process is presented in Table 4. Significant differences ($p < 0.05$) in particle concentration were observed in all confectionery products for both particles when comparing their concentrations before and after the gastrointestinal digestion process with the major influence during the salivary step according to two-way ANOVA analysis.

The interactions of (nano)particles with proteins, carbohydrates, and salts throughout the gastrointestinal tract significantly influence their stability, aggregation state and biological effects (DeLoid et al., 2017; McClements et al., 2017). The proteins present in the salivary, gastric, and intestinal fluids, can lead to adsorption onto the (nano)particle surface and the formation of proteins corona, playing a key role in modulating the ability of (nano)particles to cross lipid bilayers and interact with intestinal epithelial cells (Treuel et al., 2014). For TiO_2 nanoparticles, Ranjan et al.(2018) demonstrated, using *in silico* methods, their strong affinity for binding to proteins in the cellular environment, particularly to charged amino acid residues (Liu, Meng, Pérez-Aguilar, & Zhou, 2016). Similarly, carbohydrates can be adsorbed onto the surface of (nano)particles, forming a glycocorona (Sousa et al., 2021). These interactions could explain the results obtained in the current study. As shown in Table 3, no nanoparticulate material was detected in any of the bioaccessible extracts for TiO_2 (nano)particles. This could suggest the adsorption of TiO_2 nanoparticles may on proteins and/or carbohydrates from the gastrointestinal environment, leading to the formation of proteins corona and/or glycocoronas. However, this behavior was not observed for SiO_2 particles, likely due to differences in the intrinsic properties of the two types of (nano)particles.

As shown in Table 4, the particle percentage obtained with respect to the total amount of titanium and silicon is very low, suggesting most of titanium and silicon is present as ionic form in the extracts or as particles with a size below the LOD_{size} of TiO_2 (30–64 nm) and LOD_{size} of SiO_2 (142–151 nm). The percentage of particulate matter within this bioaccessible percentage in the intestinal stage was subsequently calculated as recommended by EFSA Scientific Committee (2018). Results in Table 4 reflected that the percentage of particles within the bioaccessible intestinal fraction for all confectionary products did not exceed 5 % for either titanium or silicon.

3.4. *Impact of in vitro digestion intestinal extracts from confectionaries on Caco-2 epithelial cells. Effect of SiO_2 and TiO_2 (nano) particles*

The results obtained in the previous studies showed the presence of a low percentage of titanium and silicon (nano)particles available for their potential absorption in the intestinal epithelium. To evaluate the extent of this absorption, the Caco-2 cell line (epithelial cells isolated from colon tissues derived from human colorectal adenocarcinoma) commonly used for performing permeability studies was selected. For this purpose, an MTT assay exposing Caco-2 cells to the *in vitro* digestion

Table 4
Concentration of TiO_2 and SiO_2 (nano)particles and particle percentages before and across the gastrointestinal digestion process.

	In vitro stage	$[TiO_2]\cdot(10^6)$ (Particle g^{-1})	TiO_2 particles (%)	$[SiO_2]\cdot(10^7)$ (Particle g^{-1})	SiO_2 particles (%)
Pure cocoa	Water	260 ± 20*	18 ± 4*	82 ± 5*	4 ± 1
	Salivary	14 ± 1	4.3 ± 0.2	13 ± 1	6.5 ± 0.8
	Gastric	14 ± 2	3.0 ± 0.1	14 ± 2	7 ± 1
	Intestinal	15 ± 1	4.5 ± 0.2	12 ± 1	4 ± 1
Custard powder	Water	503 ± 35*	17 ± 5*	59 ± 4*	5 ± 1
	Salivary	13 ± 3	3.9 ± 0.2*	4 ± 1	6.4 ± 0.4
	Gastric	5 ± 1	1.3 ± 0.4	0.35 ± 0.04*	3 ± 1
	Intestinal	2 ± 1	1.5 ± 0.3	3 ± 1	6 ± 2
Soluble cocoa	Water	170 ± 10*	21 ± 4*	11 ± 1*	1.1 ± 0.2
	Salivary	50 ± 30*	7 ± 3*	6 ± 1	1.0 ± 0.2
	Gastric	7 ± 1	1.3 ± 0.2	9 ± 1	1.1 ± 0.2
	Intestinal	7 ± 1	3.1 ± 0.5	4 ± 1	2 ± 1

* Indicates statistically significant differences ($p < 0.05$) within the same column before (water) and under *in vitro* conditions (salivary, gastric and intestinal).

intestinal extracts of confectionary products was carried out.

Due to the complexity of the gastrointestinal extracts in terms of the food matrix components and enzymes used in the different steps of the process, it would not be correct to attribute the potential cell viability decrease to the merely presence of TiO_2 and/or SiO_2 (nano)particles. To understand the effect of Ti and Si in their different chemical forms (ionic and particulate TiO_2/SiO_2) before exposing cells to gastrointestinal extracts, an MTT assay was conducted using ionic standards of titanium and silicon, anatase-phase TiO_2, and SiO_2 (nano)particles. The concentration ranges for each were chosen based on the concentrations found in intestinal fractions: ionic titanium ranging from 20-40 μg L^{-1}, ionic silicon from 200-1000 μg L^{-1}, anatase TiO_2 nanoparticles from 1-10 μg L^{-1} (10^6-10^7part g^{-1}), and SiO_2 nanoparticles from 1-10 μg L^{-1} (10^6-10^7 part g^{-1}). Due to the simultaneous presence of the ionic and particulate forms of titanium and silicon, as well as of both elements in the confectionery products, the combined effect of the different chemical forms of both elements was evaluated within the low and high ranges of each selected concentration.

According to Fig. 2 a), b), and c), no significant differences were found in the cell viability of Caco-2 cells after exposure during 4 and 24 h to the different chemical forms of titanium and silicon either together or individually.

Considering that enzymes present in intestinal extracts could contribute to the absorbance in the MTT assay, several dilution factors of 1/5, 1/10, and 1/25 of the intestinal blanks (*in vitro* digestion intestinal extracts without containing confectionary products) were applied to the cells at 4 and 24 h of exposure. The optimal dilution selected was 1/10, as it did not affect cell viability after exposure to cells for 4 and 24 h. When cells were exposed to the intestinal extracts, resulting from the *in vitro* gastrointestinal digestion process of the confectionary products, cell viability decreased more pronounced at 24 h as compared to 4 h. Comparing cell viability at both exposure times, in pure cocoa, percentages of cell viability of 83 ± 4 % and 25 ± 5 % in pure cocoa, 71 ± 6 % and 41 ± 1 %, in custard and 75 ± 2 % and 44 ± 2 % in soluble cocoa, for 4 h and 24 h of exposure, respectively were obtained all values referenced to the positive control, which exhibited 96 ± 4 % viability.

Several studies in the literature have been reported about the cytotoxicity of SiO_2 and TiO_2 (nano)particles by using different cell lines. However, no data was found in the literature regarding the exposure of Caco-2 cells to *in vitro* digestion intestinal extracts from real samples. Based on the results obtained from exposure to the standards (Fig. 2 a), b), and c)), the decrease in cell viability following exposure to intestinal food extracts (Fig. 2 d)) cannot be attributed neither to the presence of TiO_2 and/or SiO_2 particles, nor to their ionic forms (titanium and/or silicon). Therefore, this cytotoxic effect may be triggered by other endogenous components present in the matrix of confectionery

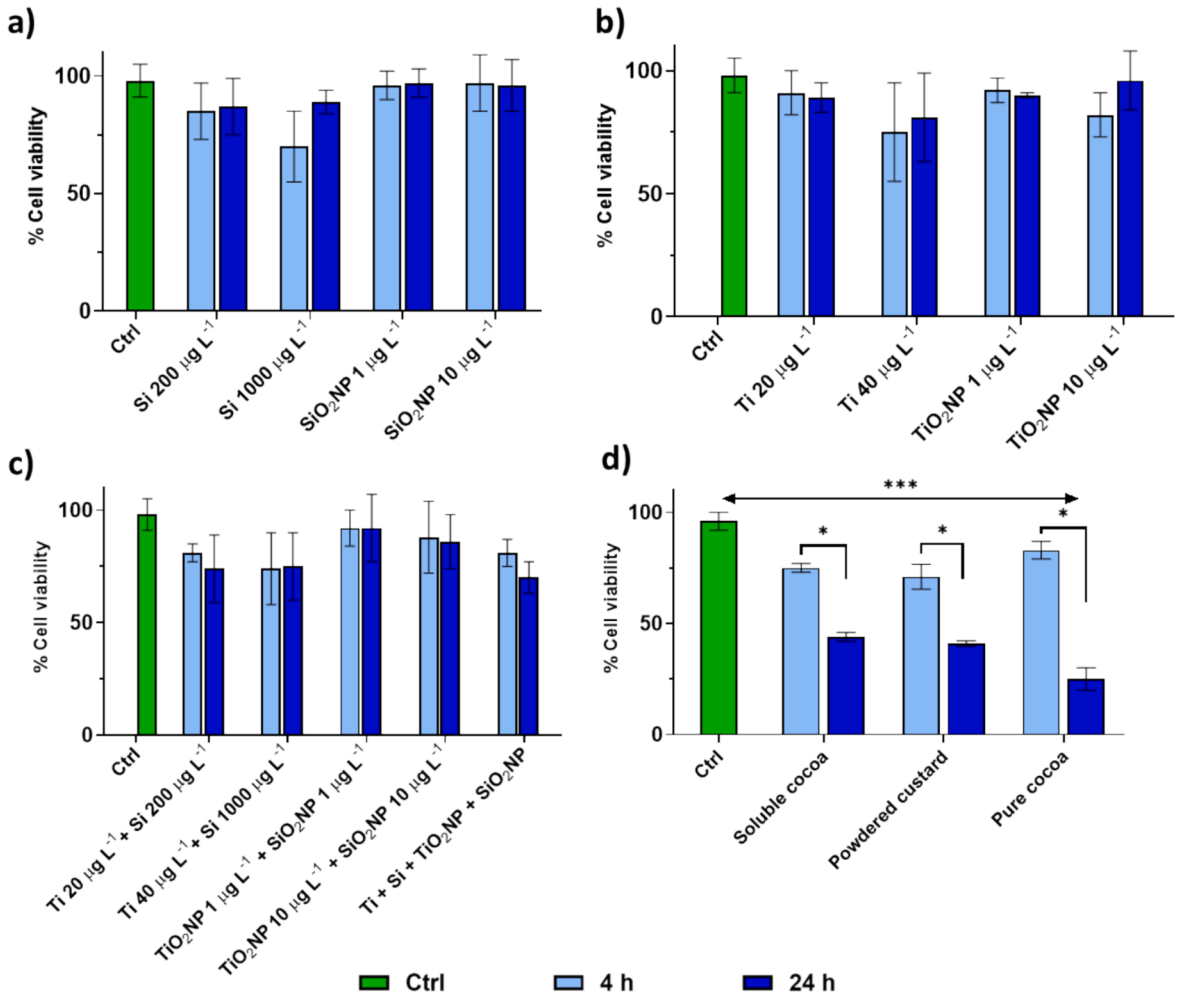

Fig. 2. Caco-2 cell viability percentage once exposed to a) ionic (titanium) and particulate (TiO_2) titanium, b) ionic (silicon) and particulate (SiO_2) silicon, c) both titanium and silicon in ionic and particulate forms, and d) soluble cocoa, custard powder and pure cocoa *in vitro* digestion intestinal extracts (diluted 1/10). Data obtained via MTT assay.

* Indicates statistically significant differences ($p < 0.05$) between 4 and 24 h of exposition time.

*** Indicates statistically significant differences ($p < 0.05$) between cells controls and intestinal food extracts from *in vitro* digestion.

products. In the present study, although particles of SiO_2 smaller than 151 nm were not detected and quantified in the intestinal extracts (LOD size by spICP-MS for intestinal stage), they could influence the cell viability. However, results in Table 4 suggest the aggregation/agglomeration of these particles during *in vitro* assay, especially during the salivary step.

Hsiao et al.(2014) conducted MTT assays to investigate how SiO_2 particle size and its concentration affected various macrophages and cell lines, including A549 (Adenocarcinoma human alveolar basal epithelial cells). They investigated exposure durations of 24 h to SiO_2 particles with sizes of 5, 60, and 200 nm, covering concentrations from 0 to 50 μg mL^{-1}. Results revealed that smaller particles exhibited higher cytotoxicity in A549 cells, and this effect was dependent on the concentration. However, for 200 nm particles, there was no significant reduction in cell viability, regardless of exposure concentration as observed in the current study with SiO_2 particles of the same size (Fig. 2a). Similar outcomes were observed by Lu et al.(2011) and Mu et al.(2012) when studying the cytotoxicity of 7–50 nm SiO_2 nanoparticles on HepG2 (Human hepatoma) and HT29 (human colorectal adenocarcinoma) cells respectively, revealing that cell viability is influenced by both dose and particle size at 24 h of exposure. However, results reported by Docter et al.(2014) highlighted a significant decrease in cell viability, due to the loss of membrane integrity, when exposing increasing concentrations of 30 nm SiO_2 nanoparticles for 4 and 24 h on the Caco-2 cell line.

The results of the present study cannot be directly compared to those obtained by Ferrante et al.(2023), who exposed increasing concentrations of TiO_2 derived from the food additive E171 to the colon cancer cell lines Caco-2 and HCT-116 for 72 h. Also, the studies conducted by Sun et al.(2017), revealed the influence of both size and morphology of TiO_2 particles of 100, 200, and 300 nm on the toxicity in the NCI-H292 Mucoepidermoid Pulmonary carcinoma cell line. The results highlighted a decrease in cell viability with increasing particle size. Additionally, the mechanism of cell death also varied depending on the particle size. It should be noted that the aforementioned studies have not been conducted with standard suspensions of particulate material alone without considering the effect of food matrix, where (nano)particles are presented.

4. Conclusions

The evaluation of the risk associated with nanoparticle forms in food additives, such as TiO_2 (E171, banned in Europe) and SiO_2 (E551), requires in-depth knowledge of their physicochemical properties before and after oral ingestion and their potential to induce cytotoxic effects. Results from the application of an *in vitro* gastrointestinal assay from the selected confectionary products revealed no significant changes in size distribution throughout the *in vitro* gastrointestinal process. TiO_2 nanoparticles were detected at concentrations below 50 % of total Ti concentration. The percentages of bioaccesibility found for TiO_2 and SiO_2 were very low not exceeding 5 % and 6 %, respectively. The cytotoxicity results showed a decrease in Caco-2 cell viability after 24 h of exposure to intestinal extracts from confectionery products, indicating a cytotoxic effect. However, this effect was not due to the presence of (nano)particles themselves but to the components of food matrix. While the bioaccessible concentrations of particles alone did not significantly affect cell viability, the full gastrointestinal extracts caused a substantial decrease, likely due to other food constituents.

These findings highlight the importance of including food matrices in cytotoxicity studies, as the mere use of pristine (nano)particles standard solutions may overestimate their effects and underestimate the influence of food components. This approach provides a more realistic assessment of their impact on real-world dietary scenarios. This study is the first reported in the literature that considers the combined impact of TiO_2 and SiO_2 (nano)particles and food matrix.

CRediT authorship contribution statement

Elena Espada-Bernabé: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Gustavo Moreno-Martín:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **Beatriz Gómez-Gómez:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Yolanda Madrid:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.115754.

Data availability

Data will be made available on request.

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