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Development of a fast and low-cost aqueous based-extraction protocol for the simultaneous extraction and characterization of SiO_2 and TiO_2 (nano) particles in confectionary products

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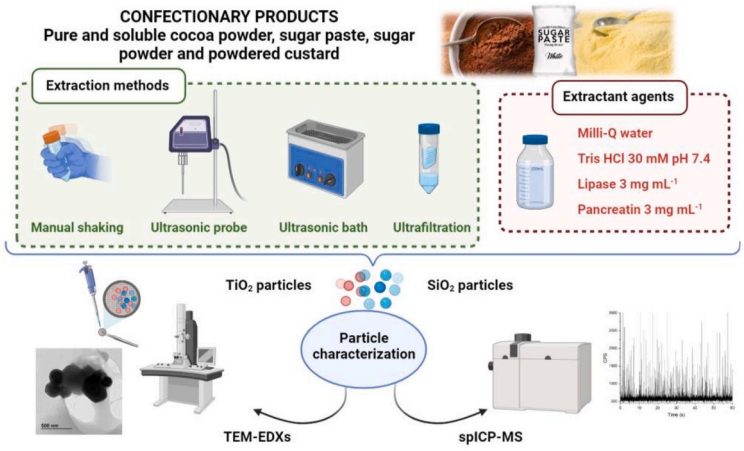
HIGHLIGHTS

- Simultaneous extraction protocol for TiO_2 and SiO_2 particles has been optimized.
- TiO_2 and SiO_2 (nano)particles were detected for all the evaluated confectionaries.
- E171 and E551 did not appear on the labeling of most confectionary products.
- High TiO_2 nanoparticle content was detected for soluble cocoa and powdered sugar.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: The determination of (nano)particulate content from food additives has been a long-standing concern for authorities since it is of vital importance for ensuring food safety, regulatory adherence, and transparent consumer information. Nonetheless, a critical step in these determinations is the refinement of a careful and quantitative extraction process for particles that may be found within complex matrices such as confectionery products. The development of new technologies and analysis methods for nanoparticles is ongoing. Whereas new technologies and analysis methods for nanoparticles are being developed, the extraction of (nano) particles of different nature has not been adequately addressed in the literature.

Results: A simple aqueous extraction procedure was found to be suitable for the simultaneous extraction of TiO_2 and SiO_2 (nano)particles from five confectionery products. Neither the extraction agents (water, lipase, pancreatin and Tris-HCl solutions) nor the methods (manual shaking, ultrasonic bath, ultrasonic probe and ultrafiltration) altered the size, morphology, or aggregation state of either type of particle, as revealed by the

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micrographs obtained by Transmission Electron Microscopy (TEM). Single-particle ICP-MS (spICP-MS) determined that the optimal conditions for extracting both types of particles involve manual shaking using water as the solvent. Furthermore, the use of enzymes seemed to hinder the determination of both types of particles by spICP-MS. (Nano)particles of TiO_2 and SiO_2 were detected in all the confectionaries, even though the E171 additive was only labeled in one of them. The average percentage of nanoparticulate TiO_2 material in the evaluated products was 30 %, while no nanometer-sized particles of SiO_2 were detected.

Significance: Ensuring food safety, regulatory compliance and transparent consumer information relies on getting reliable results that connect with the application of sample treatment procedures for detecting unaltered nanoparticles in food products. The presented research introduces an economical, swift, user-friendly, environmentally responsible, and harmonious extraction method for the concurrent analysis of TiO_2 and SiO_2 particles in confectionery samples. Furthermore, particles from additives not included in the labeling have been detected, characterized, and quantified in the confectionary products.

1. Introduction

The use of certain additives in the food industry has been called into question by the competent authorities in terms of safety, since the information provided in scientific reports, assessed according to existing guidance for evaluating possible risks related to the presence of nanoparticles [1,2], raises doubts about their possible adverse health effects for humans. Even though regulations stipulate that the utilization of engineered nanomaterials in food products, should be listed as ingredients, however this information is frequently omitted.

Two of the most controversial additives worldwide are the food grade TiO_2 (E171) and SiO_2 (E551). Both have been re-evaluated several times to ensure their use is safe in the light of current evidence. In both cases, during the manufacturing process of the additive itself, the formation of TiO_2 or SiO_2 nanoparticles occurs spontaneously.

TiO_2 is widely used as whitening agent in confectionery, cosmetics and other products [3–5]. In the EU, based on the re-assessment of E171 safety as a food additive carried out by EFSA in 2021, the use of food grade titanium dioxide has been banned in food [6], while in countries such as the United States it is still permitted. E171 is found in the form of a polydisperse substance consisting mainly of anatase particles, although rutile particles are sometimes present. The constituent sizes of particles typically vary from 30 to 350 nm, with smaller or larger particles being relatively rare [7–10].

On the other hand, food-grade silica is used as an anti-caking agent in powdered products such as toothpaste or confectionaries. The safety of E551 as a food additive is currently being re-assessed in the EU [11]. E551 is characterized by the presence of small constituent particles, typically measuring less than 20 nm in diameter. These individual particles usually form small aggregates. These constituent particles, along with the clusters, combine to create agglomerates with a fractal-like structure.

For a correct physicochemical characterization of nanoparticles it is necessary to use complementary techniques that provide a global view of the different parameters to be determined [12,13]. Among these techniques are electron microscopy (Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) coupled with Energy Dispersive X-Ray (EDX)) and light scattering techniques (Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA), Multi Angle Light Scattering (MALS)). Also, separation techniques such as Field Flow Fractionation (FFF) coupled with DLS, MALS and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) have been used for characterization purposes. ICP-MS in single particle detection mode (spICP-MS) is nowadays considered as a powerful tool for the determination of the concentration and size distribution of inorganic (nano)particles [14]. In addition, the usefulness of spICP-MS for the analysis of particles such as TiO_2 and SiO_2 in various types of samples has been reported [15,16]. A recent interlaboratory exercise has shown that spICP-MS and TEM can be used for screening and verifying the presence of E171 in confectionery products, respectively [17–20].

Sample treatment is considered the limiting step when performing nanoparticles characterization in complex samples [21]. In order to ensure accurate results, it is essential to keep unaltered the original nanoparticles physicochemical properties during extraction, as well as to avoid any undesired interactions between nanoparticles and other components in the extraction matrix [22].

For this objective, various authors have assessed diverse methods to extract nanoparticles from confectionery products. These methods include applying mild oxidation conditions [23,24], dissolving the product in ultrapure water followed by sonication in an ultrasonic bath [25], and using enzymes like proteinase K and α-amylase [21,26]. In addition, extraction conditions such as temperature and time must be controlled carefully. Most of the developed sample treatment methods are focused on the extraction of a single type of nanoparticle and very few of them have paid attention to the simultaneous extraction of different types of nanoparticles from the same sample as the simultaneous isolation of TiO_2 and SiO_2 nanoparticles in food and confectionery products.

The primary aim of this study is to explore the potential presence of TiO_2 and SiO_2 (nano)particles originating from food additives in powdered confectionery products. To achieve this, a method has been designed to extract simultaneously both types of particles while maintaining their integrity in the confectionary products selected. After optimizing the extraction protocol, the particles extracted from different powdered confectionery products were identified, quantified, and characterized using TEM and spICP-MS.

2. Materials and methods

2.1. Samples

In this work, different confectionary products, pure cocoa powder, soluble cocoa powder, powdered custard ~~powdered~~, sugar paste and powdered sugar, that may contain TiO_2 or/and SiO_2 (nano)particles as additives E171 and E551 have been studied. The additive E171 is listed as an ingredient only in the case of sugar paste.

The ingredients list for each confectionery product is shown in the SM.

All products were obtained from supermarkets in the Community of Madrid (Spain).

2.2. Total Ti and Si content determination in confectionary products

To assess total Ti and Si concentration in the confectionary products, samples were subjected to microwave-assisted mineralization. Each sample was weighed (100.0 mg) in triplicate in a double-wallet advanced composite vessel and 4.4 mL of a mixture of 3.6 mL 65 % (v/v) nitric acid (Merck, Spain), 0.6 mL 30 % (v/v) hydrogen peroxide (Panreac, Spain) and 0.2 mL 47–51 % (v/v) hydrofluoric acid (Fluka Analytical, Spain) were added to each vessel. Total mixture was submitted to a microwave heating program consisting of 20-min ramp to 210 °C and 10-min hold time. Blank solutions were also performed. The digested extracts were transferred to 50 mL disposable polypropylene tubes and brought the total volume until 25.0 mL with Milli-Q ultrapure

water. Finally, samples were conveniently diluted with Milli-Q water before ICP-MS analysis.

Ti and Si contents in the digests were measured by ICP-MS (Agilent 7700x, Agilent Technologies Inc., USA) monitoring ^{48}Ti and ^{28}Si under continuous acquisition mode. Nebulization gas flow, torch position, optical lenses and quadrupole voltages were daily optimized. In an attempt to improve sensitivity for Si, hydrogen as collision gas was evaluated, but no significant improvements were achieved [27].

Operational conditions and data acquisition parameters for ICP-MS measurements are shown in Table 1.

Due to the complexity of sample matrices involved in this study, standard addition calibration was performed to assess whether samples have matrix effects. The slopes of the addition calibration curves for each sample were compared to that obtained for the external calibration curve by means of a Student t-test ($\alpha = 0.05$). No matrix effect was found for any of the samples analyzed. Consequently, external calibration was used for Ti and Si in ranges of 0–75 μg L^{-1} and 0–500 μg L^{-1} in Milli-Q water, respectively using a Ti and Si ionic standard solutions for ICP-MS (Merck, Spain).

The detection and quantification limits for both elements were calculated from the blanks of the respective external standards, as no matrix effect was observed for any of the products regarding Ti or Si. For Ti, a LOD of 6 μg g^{-1} and LOQ of 19 μg g^{-1} were obtained. For Si, these values increased to a LOD of 97 μg g^{-1} and LOQ of 324 μg g^{-1}. The detection limits were calculated as three times the blank standard deviation, while the quantification limit was determined as ten times the blank standard deviation [28]. Sample preparation and dilutions were considered for this calculation.

Table 1
Operating conditions for ICP-MS and spICP-MS.

ICP-MS parameters	
RF Power (W)	1550
Plasma gas flow rate (L min^{-1})	12.0
Ar auxiliary flow rate (L min^{-1})	0.90
Nebulizer	MicroMist
Carrier gas flow rate (L min^{-1})	1.1 for ICP-MS 0.68 for spICP-MS
Torch (internal diameter in mm)	2.5 for ICP-MS 1.0 for spICP-MS
Spay chamber	Scott
Data acquisition parameters for ICP-MS	
Measuring mode	Spectrum
Monitored isotopes	^{28}Si, ^{48}Ti
Data acquisition parameters for spICP-MS	
Measuring mode	Single particle detection in TRA mode
Dwell time (ms)	3
Acquisition time (s)	60
Flow rate (mL min^{-1})	0.30 ± 0.02
Monitored isotopes	^{28}Si, ^{48}Ti

2.3. Optimization of TiO_2 and SiO_2 particles extraction from confectionary products

Different extracting agents (aqueous or enzymatic) and processes (manual shaking and ultrasonic bath or probe) were evaluated for isolating TiO_2 and SiO_2 particles from the confectionary products investigated in this study. For this purpose, Milli-Q water, Tris-HCl (30 mM, pH 7.4), lipase and pancreatin were tested as extraction agents under different conditions: manual shaking (10 min), ultrasonic bath (Elmasonic S 60, operating at a frequency of 37 kHz of frequency during 10 min) and ultrasonic probe (BANDELIN SONOPULS HD 3200) by applying cycles of 0.5 s during 10 min at 60 % of ultrasound amplitude, maximum amplitude 20 kHz and maximum power of 200 W) (Table 2).

With the aim of elucidating possible changes on nanoparticles size, aggregation/agglomeration state or dissolution as consequence of the use of the different extractants and extraction processes, the different extraction procedures were tested first by using standards suspensions of 25 nm TiO_2 anatase nanoparticles standard (Merck, Spain) and 80 nm Silica Nanospheres (nanoComposix, California) at a concentration level of 10 mg mL^{-1} standard. For this purpose, SiO_2 nanoparticles standard were diluted in the proposed extractant agents and submitted to manual shaking, ultrasonic bath or ultrasonic probe by applying the conditions listed in Table 2. TiO_2 anatase phase nanoparticles were also dispersed in the same agents and submitted to manual shaking and ultrasonic bath. It is important to note that, as the ultrasonic probe is composed of Ti, this extraction method was not applied for TiO_2 particles extraction. On the other hand, nanoparticles in contact with enzymes (lipase and pancreatin) were incubated overnight at 2.8 g (200 rpm) in a horizontal shaking incubator at 37 °C [22] before applying manual shaking or ultrasonic energy (Table 2). After that, changes in nanoparticles properties were evaluated using TEM as described in Section 2.4.

Once it was verified that the extraction process did not affect the morphology, size, aggregation/agglomeration state and dissolution of TiO_2 and SiO_2 nanoparticles, the different extraction agent and conditions were tested in cocoa powder samples selected as an example of food matrix. For this purpose, approximately 0.050 g of confectionary product were suspended up to a 30.0 mL with the different extractant agents in 50 mL polypropylene tubes and subjected to the different conditions described in Table 2. Extracts were characterized in terms of size, size distribution, morphology, agglomeration state and particle concentration by TEM and spICP-MS following the procedure described below.

2.4. Physicochemical particle characterization

2.4.1. TEM analysis

Particle characterization in terms of constituent particle size, morphology, aggregation/agglomeration state and composition were carried out by TEM and EDX with a JEOL JEM 1400 PLUS electron microscope operating at 140 kV and equipped with a charge-coupled device CCD camera (KeenView Camera) (JEOL Ltd., Tokyo, Japan). Samples for TEM were prepared by placing a few drops of each extract on copper grids and left to dry under ambient temperature before TEM analysis. To avoid the occurrence of agglomerates, samples were

Table 2
Sample extraction protocols applied.

Extraction method	Time (min)	Extraction agents*	Particle
Manual shaking	10	Milli-Q water, Lipase (3 mg mL^{-1}), Pancreatin (3 mg mL^{-1}), Tris-HCl (30 mM, pH 7.4)	TiO_2, SiO_2
Ultrasonic bath	10		TiO_2, SiO_2
Ultrasonic probe	10		SiO_2

*Every extraction agent was evaluated under every single extraction method.

dispersed in an ultrasound bath during 5 min before analysis before drop deposition. Moreover, Image J open-source software was used to obtain constituent particle size and size distribution from TEM images according to counting rule number 4 as defined in Bresch et al., 2022 [29].

2.4.2. spICP-MS analysis

Particle size distribution and concentration (part L^{-1} or part g^{-1}) of TiO_2 and SiO_2 (nano)particles isolated from confectionary products were determined by spICP-MS. Default instrumental and data acquisition parameters have been gathered in Table 1.

High dilutions rates for samples had to be applied before spICP-MS measurement to achieve low ^{48}Ti and ^{28}Si ionic background signal, as well as to guarantee that only one particle reaches the detector per dwell time interval. Moreover, for each sample, data were acquired, using the parameters indicated in Tables 1 and in triplicate for both ^{48}Ti and ^{28}Si isotopes separately with the aim of obtaining an appropriate number of events (between 600 and 1200). Transport efficiency was daily calculated by using a diluted solution of a quality control material LGCQC5050 (citrate-AuNPs) of known size (30 nm) and concentration (mass concentration of 20 ng L^{-1}/particle number concentration of $7.3{\cdot}10^7$ NP L^{-1}) by monitoring ^{197}Au signal. Calibration curve of ionic Ti and Si ranging from 0 to 75 μg L^{-1} and 0–500 μg L^{-1} respectively were prepared daily by employing ionic standard solutions for ICP-MS in Milli-Q water.

Raw signal intensity data were treated manually as described by Peters et al. [30], by using the free RIKILT spreadsheet for Microsoft Excell obtained from the website http://www.wageningenur.nl/en/Expertise-Services/Research-Institutes/rikilt/So?ware-and downloads.htm. Particles were discriminated from the background signal using a 5-σ criterion approach (σ: standard deviation of the baseline), aiming to reduce the number of false positive particle detection [31,32].

Transport efficiency was calculated according to the particle frequency method developed by Pace et al., 2011 [33]. For obtaining TiO_2 particle size a ratio between molar mass of the particle material and molar mass of the analyte being measured of 1.667 and density of 3.9 g cm^{-3} were considered, due to anatase is the most commonly used crystalline form for food-grade TiO_2 [34]. In case of SiO_2, this ratio was set at 2.143 using 2.6 g cm^{-3} as density value. For spICP-MS calculations it was assumed that TiO_2 and SiO_2 (nano)particles were spherical. The flow rate of 0.30 ± 0.02 mL min^{-1} was calculated daily as the variation in mass of Milli-Q water introduced into the equipment over a period of 15 min.

To evaluate the accuracy of the developed method, the particle size and number concentration of three individual AuNPs LGCQC5050 suspensions were determined and compared with the certified values. No statistically differences ($p > 0.05$) were obtained between the values determined by spICP-MS and those certified (data not shown). These measurements were repeated in different days providing similar results.

In the case of sugar paste, ionic and particulate Ti were separated using an ultrafiltration process with Amicon® Ultra-4 Centrifugal Filter Units (3 kDa) [15] before spICP-MS analysis. Specifically, 3.000 g of sample was weighed into 50 mL polypropylene tubes and dispersed in 40.0 mL of Milli-Q water via sonication in an ultrasonic bath. Then, 4.0 mL of the solution was centrifuged at 7500 g for 1 h. The particles retained in the filter were then resuspended in polypropylene tubes. Both the particulate (top layer) and ionic (bottom layer) fractions were stored at 4 °C until spICP-MS analysis.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was applied as statistical tool to compare variances across the means (or average) of different groups. A significance level of p-value < 0.05 was adopted for all comparisons. Statistical analysis of data was carried out using Statgraphics Centurion XIX (Statgraphics Technologies Inc., USA).

3. Results and discussion

3.1. Total Ti and Si content

Total content of Si and Ti was determined in the confectionary products selected for this study by ICP-MS after an acid mineralization process to elucidate the possible presence of TiO_2 and SiO_2.

The determination of Ti an Si by single quadrupole ICP-MS is not straightforward. It is well documented in literature [7,35] that the ^{48}Ti isotope, which is the most abundant Ti isotope (73.73 %) is affected by the presence of polyatomic interferences from $^{32}S^{16}O$ and $^{36}Ar^{12}C$, and the isobaric interference of ^{48}Ca. Therefore, ^{48}Ca (0.187 %) was also monitored in the confectionary products. No significant differences were observed between blanks and samples when registering the ^{48}Ca signal, suggesting that Ca level in the samples is not high enough to affect Ti signal.

Interferences when measuring Si in a single quadrupole ICP-MS are extensively reported in literature [27,36]. The most abundant ^{28}Si isotope (^{28}Si, with 92.23 % of relative isotopic abundance) is interfered with polyatomic species (such as $^{12}C^{16}O$ and $^{14}N_2^+$) [36]. To achieve lower background signals, the silicon isotope m/z 29 (4.67 % relative isotopic abundance) is commonly monitored. In this study, using an ICP-MS with a single quadrupole, both silicon isotopes m/z 28 and 29 were monitored, both with and without a gas cell (H_2). No significant improvements in sensitivity were observed for either isotope under both non-gas and gas modes. Therefore, ^{28}Si (most abundant silicon isotope) was monitored in this study in non-gas mode (LODs of 40 and 39 μg L^{-1} for non-gas and gas modes, respectively, were obtained).

Both Ti and Si were detected in all analyzed confectionery products, as it is indicated in Table 3. Similar Ti contents were found in pure cocoa powder, soluble cocoa powder, powdered custard, and sugar glass (Table 3). A significantly higher Ti content was found in sugar paste, likely the reason it is labeled as food-grade additive E171 in the ingredient list. The characteristic white color of sugar paste may be attributed to the addition of E171.

On the other hand, Si contents were quite dissimilar among the different products considered presenting in general higher amounts compared to Ti contents. However, additive E551, or Si in its individual form, was not included in the list of ingredients in any of the analyzed products. Furthermore, it is noteworthy that silicon ranks as the second most prevalent element in the Earth's crust, thus its presence in elevated concentrations is to be expected.

Therefore, the addition of E171 or E551 cannot be excluded based on the total Ti or Si analysis (Table 3). Consequently, analytical determinations of TiO_2 and SiO_2 particulate content in these foods are necessary.

3.2. Optimization of extraction process for isolating TiO_2 and SiO_2 particles

Particle extraction procedures have been reported to be the critical step in sample preparation for particle analysis in food samples. Therefore, different extraction methods (aqueous or enzymatic) and processes (manual shaking and ultrasonic bath or probe) were investigated for isolating TiO_2 and SiO_2 particles from the confectionary

Table 3
Total Si and Ti content in the confectionary food products obtained 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
Sugar paste	5300 ± 250	750 ± 63
Sugar powder	1791 ± 39	78 ± 1

All data expressed as average ± SD of three independent samples.

products (Table 2). Manual shaking and ultrasonic bath were employed with specific extraction agents such as Milli-Q water, Tris-HCl (30 mM, pH 7.4), Lipase (3 mg mL^{-1}) and Pancreatin (3 mg mL^{-1}), targeting both TiO_2 and SiO_2 particles. The ultrasonic probe was also employed for assisting the extraction processed with the same extraction agents but only in the case of SiO_2 particles.

For the selection of extraction agents several factors were considered but with the aim of achieving simultaneous extraction of both types of particles. Firstly, the selected extractant should keep the initial morphology of the particles, preventing also their dissolution. Based on that acidic extraction was avoided as reported by Laborda et al., 2016 [12].

Secondly, considering the samples' high sugar and fat content [15], enzymatic agents capable of degrading these components were selected [21,30,37]. Given that the selected confectionary products contain carbohydrates and fats, lipase was chosen to break down lipids, while pancreatin was selected to decompose carbohydrates and fats.

3.2.1. *Stability of TiO_2 and SiO_2 nanoparticle standards under different extraction conditions*

The first step in the optimization of the extraction process was to evaluate the possible changes in particles size, morphology, and aggregation/agglomeration state that TiO_2 and SiO_2 particles may undergo under the different extracting agents and conditions studied. As a first step extracting agents and extraction protocol were applied to TiO_2NPs and SiO_2NPs standard suspensions. Therefore, 80 nm SiO_2 and 25 nm TiO_2 anatase-phase standard suspensions were characterized by TEM after being subjected to the different extraction conditions.

TEM micrographs in Fig. S1 and Fig. S2 evidence no effect of extraction conditions on the morphology and agglomeration state of 80 nm SiO_2 and 25 nm anatase-phase TiO_2 nanoparticles, respectively. Furthermore, Table S1 gathered exact particle sizes calculated from TEM images by using ImageJ free software. From Table S1, it can be concluded that the particle sizes for both 80 nm SiO_2 and 25 nm anatase-phase TiO_2 nanoparticles were similar, regardless of the extraction agent or method used. A one-way ANOVA analysis showed no significant differences in particle sizes across the different treatments. Based on the micrographs depicted in Fig. S1 and Fig. S2 and Table S1, it can be concluded that the morphology and agglomeration state of both types of particles remains unaffected by the various extraction conditions applied.

3.2.2. *Evaluation of the optimal extraction condition for TiO_2 and SiO_2 particles from food matrix*

After demonstrating that TiO_2 and SiO_2 particles remained unaltered by the evaluated extraction conditions, the extraction efficiency of different agents and processes was investigated using pure powdered cocoa as a food matrix example.

Fig. S3 shows, as an example, TEM micrographs of the extracts obtained from pure powdered cocoa sample after carrying out an aqueous or enzymatic extraction. However, the limited number of particles detected by TEM as well as the unfeasibility of distinguishing between TiO_2 and SiO_2 particles hampered the acquisition of quantitative size values from images. Thus, to gather quantitative data concerning potential changes in particle concentration and size for both additives after applying the different extraction conditions, extracts were analyzed by spICP-MS.

Table 4
Size and particle number detection limits for TiO_2 and SiO_2 in spICP-MS.

Extractant agent	LOD_{size} TiO_2 (nm)	LOD_{NP} TiO_2 (NP L^{-1})	LOD_{size} SiO_2 (nm)	LOD_{NP} SiO_2 (NP L^{-1})
Water	30 ± 5	(3.2 ± 0.7)· 10^6	142 ± 15	(2.5 ± 0.4)· 10^8
Tris-HCl	32 ± 3		154 ± 20	
Lipase	54 ± 9		176 ± 28	
Pancreatin	52 ± 9		173 ± 30	

High background levels were detected when measuring real confectionery product samples by spICP-MS, due to the complexity of the samples and the inherent interferences of the elements analyzed. As described in Section 2.4.2, to accurately discriminate between the ionic background and particles, the 5-σ criterion was applied [31,32]. Consequently, size detection limits (LOD_{size}) and particle number detection limits (LOD_{NP}) were calculated for both particle types across various extraction agents (Table 4). As it can be seen in Table 4, the detection limits increased when using enzymatic agents (lipase and pancreatin) for both particle types, resulting in higher signal backgrounds. This poses a limitation for quantifying smaller particles, which could be erroneously classified as ionic material.

For Ti determination by spICP-MS, as for conventional ICP-MS measurements (Section 3.1.), the potential for isobaric interferences between ^{48}Ti and ^{48}Ca were considered. Thus, ^{48}Ca were also monitored in the samples analyzed by spICP-MS in which low levels of this element were found as part of the background (data not shown). Based on that, extracts were analyzed by spICP-MS in no gas mode since the low levels of ^{48}Ca present in the samples resulted in a continuous background from which ^{48}Ti spikes can be differentiated. Bucher and Auger, 2019 [6] addressed this interference when measuring TiO_2 particles using spICP-MS in food products containing E171, concluding that such interference can be disregarded in these samples, even without utilizing a collision cell. Also Geiss et al. (2021) [17], when measuring, by spICP-MS, various titanium isotopes in confectionery samples containing E171, concluded that the presence of calcium, coupled with its low isotopic abundance (0.187 %), poses no issue.

Under the conditions described above, the LOD_{size} calculated for TiO_2 particles varies between 30 and 52 nm depending on the extractant agent, as it can be seen in Table 4. The range of detection limits for this isotope, ^{48}Ti, aligns with those found by Givelet et al., 2021 [38] when analyzing analgesics (LOD size <30 nm) and chewing gums (LOD size 29–56 nm). Similar results were found by Lee et al., 2014 [39] yielding an LOD size in the range of 21–80 nm.

In relation to Si measurements there were difficulties to distinguish background from particle signal due to the interferences of ^{28}Si described above (Section 3.1) [27,36]. Once again, the employment of H_2 as collision gas was tested but levels of background were similar to that obtained monitoring ^{28}Si in no gas mode. Recommendations based on measuring protocol from Bolea-Fernández et al. [36], were applied to obtain a background signal in the range of 0.1–5 μg L^{-1} of ionic ^{28}Si. Consequently, high dilution factors were applied to samples. LOD_{size} for SiO_2 particles in spICP-MS were estimated to be over 142 nm, depending on the extractant agent, as can be seen in Table 4. The size detection limit for SiO_2 was slightly lower than those reported in the literature [30,39], which were closer to 200 nm under the same measurement conditions as in the current manuscript.

Taken all these observations into account, the extracts resulting from applying the different extraction conditions were analyzed by spICP-MS monitoring ^{48}Ti and ^{28}Si in two different runs. Table 5 shows data about percentage of SiO_2 and TiO_2 particles and total Si and Ti extracted, as well as mean particle sizes (equivalent spherical diameter).

A one-way ANOVA analysis revealed no statistically significant differences in particle size among the various physical extraction methods for SiO_2 (manual shaking, bath, and ultrasonic probe) and TiO_2 (manual shaking and ultrasonic bath). Similarly, no significant differences were found when comparing the extraction agents. Therefore, results seem to indicate that neither the extractants' nature nor the extraction process has an influence on particle size of the extracted particles that may be presented in the sample analyzed.

However, statistically significant differences (p-value < 0.05) were found after performing one-way ANOVA analysis among percentages/concentration (number-based) of particles and total Si and Ti extracted (Table 5). Percentage of particles (% part) were calculated by dividing the mass particle concentration obtained by spICP-MS by total Si or Ti

Table 5
SiO_2 and TiO_2 particle concentration and percentage, average size, total Si and Ti extracted under the different extraction conditions on pure powdered cocoa samples.

Extractant agent	Extraction method	SiO_2				TiO_2			
		% part	% Total Si	Mean size (nm)	Particle concentration·10^8 (Part g^{-1})	% part	%Total Ti	Mean size (nm)	Particle concentration·10^8 (Part g^{-1})
Water	Manual shaking (10-min)	4 ± 1	95 ± 2	269 ± 27	8.2 ± 0.5	18 ± 4	71 ± 6	126 ± 40	2.6 ± 0.2
	U-bath (10-min)	8 ± 1	79 ± 1	273 ± 30	3.9 ± 0.4	18 ± 3	81 ± 8	133 ± 40	3.1 ± 0.3
	U-probe (10-min)	7 ± 1	87 ± 1	277 ± 38	8.6 ± 0.9	–	–	–	–
Tris-HCl	Manual shaking (10-min)	5 ± 1	97 ± 1	267 ± 29	6.4 ± 0.4	21 ± 1	74 ± 6	135 ± 48	2.8 ± 0.1
	U-bath (10-min)	10 ± 1	82 ± 1	271 ± 30	8.1 ± 0.3	21 ± 4	80 ± 5	140 ± 44	3.2 ± 0.2
	U-probe (10-min)	12 ± 0	75 ± 1	271 ± 32	9.0 ± 0.2	–	–	–	–
Lipase	Manual shaking (10-min)	8 ± 1	77 ± 1	265 ± 32	8.6 ± 0.6	6 ± 1	195 ± 2	121 ± 30	2.3 ± 0.3
	U-bath (10-min)	10 ± 1	73 ± 1	267 ± 31	8.7 ± 0.5	5 ± 2	190 ± 6	116 ± 31	3.2 ± 0.3
	U-probe (10-min)	10 ± 1	64 ± 1	275 ± 30	7.9 ± 0.5	–	–	–	–
Pancreatin	Manual shaking (10-min)	8 ± 1	77 ± 1	267 ± 31	6.8 ± 0.4	10 ± 2	142 ± 6	129 ± 41	3.4 ± 0.2
	U-bath (10-min)	13 ± 1	74 ± 1	273 ± 30	8.0 ± 0.5	13 ± 2	165 ± 17	131 ± 42	3.8 ± 0.2
	U-probe (10-min)	11 ± 1	78 ± 1	274 ± 30	9.0 ± 0.6	–	–	–	–

content found in pure powdered cocoa analyzed by ICP-MS after acid digestion (total content reported in Table 3). Likewise, percentages of total Si or Ti extracted were calculated dividing the total Si or Ti obtained by spICP-MS (sum of particulate and ionic Si or Ti) by total Si or Ti determined by ICP-MS after acid digestion. In case of SiO_2, higher percentages of particles (also particle concentration) and total Si extracted were obtained using aqueous extractions rather than employing lipase and pancreatin. Nevertheless, similar values were obtained for these parameters regardless of the physical extraction process used. Similar conclusions can be drawn for TiO_2, as better results were achieved with aqueous extraction, and there were minimal differences between using manual shaking or an ultrasonic bath to assist the extraction process. It is important to note that higher background levels were observed for lipase and pancreatin when monitoring titanium in spICP-MS mode compared to Milli-Q water. Additionally, Tris-HCl resulted in larger size and particle number detection limits, thereby limiting the detection of smaller particles, which may be quantified as ionic material. This likely explains why the percentage of total Ti was well above 100 %.

It is important to highlight that no TiO_2 or SiO_2 particles were detected in the blanks of the various extraction agents employed. However, when monitoring both elements, higher ionic backgrounds were observed with the use of enzymatic agents (lipase and pancreatin), as indicated by the particle size detection limits shown in Table 4.

Therefore, given the provided data and focusing on the simultaneous determination of TiO_2 and SiO_2 particles in confectionery samples through a unified extraction protocol, water as the solvent and manual shaking as the extraction method were chosen as optimal. This selection simplifies sample processing steps and offers a more cost-effective and environmentally friendly solvent for extracting SiO_2 and TiO_2 particles in such samples. Additionally, employing enzymes as extraction agents in the assessed scenarios had highlighted challenges in spICP-MS measurements.

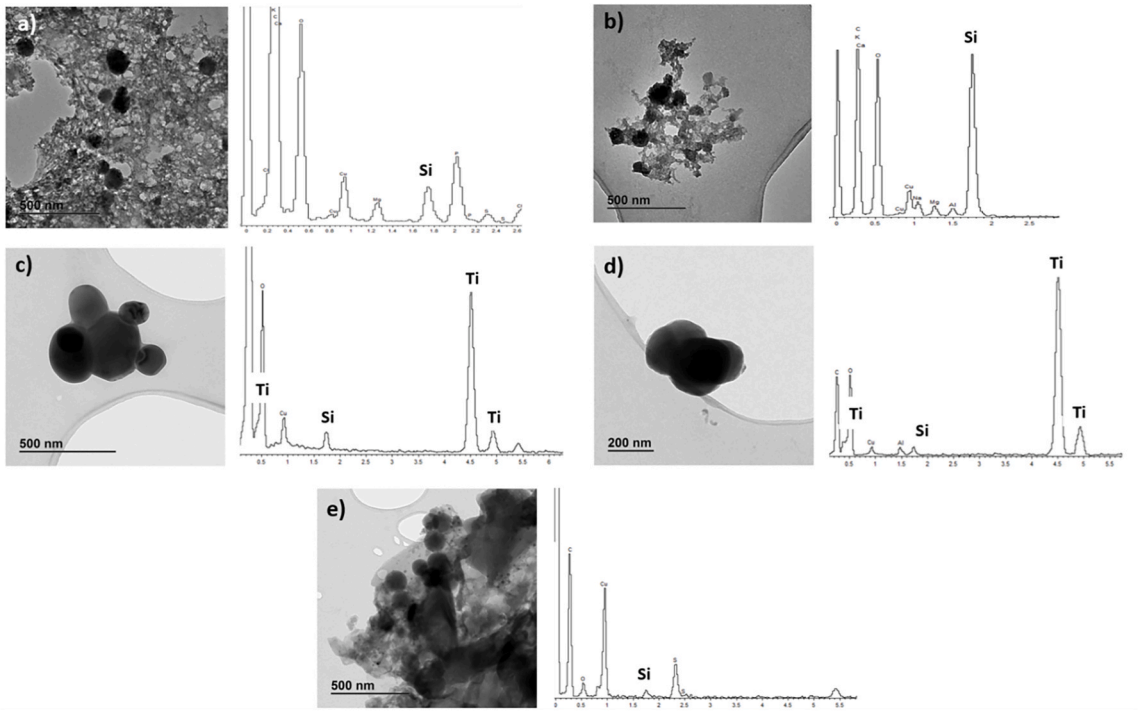

Fig. 1. TEM micrographs and EDX spectra of TiO_2 and SiO_2 (nano)particles from a) powdered cocoa, b) soluble cocoa, c) sugar paste, d) powdered custard and e) sugar powder under optimal extraction conditions.

3.3. Identification, characterization, and quantification of TiO_2 and SiO_2 (nano)particles in confectionary products

Under optimal conditions described in Section 3.2, TiO_2 and SiO_2 particles were extracted from pure and soluble cocoa powder, powdered custard, sugar paste, and sugar powder using water as the extractant agent and manual shaking as the extraction method. The extracted solutions were then analyzed and characterized using TEM and spICP-MS.

TEM micrographs showed in Fig. 1 evidence the presence of agglomerated particles with polygonal shapes and curved ends in all the samples studied. Moreover, EDX analysis evidenced the presence of Ti (Kα (4.508 eV) and Lα (0.452 eV)) and Si (Kα (1.740 eV)) in the composition of the particles detected (Fig. 1). However, Ti was only detected in sugar paste and powdered custard, whereas Si seemed to be present in all the confectionary products analyzed: powdered cocoa, soluble cocoa, sugar paste, powdered custard, and sugar powder.

Nevertheless, once again, the low number of particles detected made it difficult the acquisition of quantitative data from TEM images. Moreover, the presence of both elements in EDX spectra hinders getting reliable information of both particles individually. Also, it should be noted that these are food samples with complex matrices that make it difficult to visualize particles by TEM. Therefore, extracts were further analyzed by spICP-MS.

Significant spikes clearly differentiated from background signal for Ti and Si were registered when analyzing extracts from powdered cocoa, soluble cocoa, sugar paste, powdered custard, and sugar powder, whereas no signals were detected in blanks. As comparative example, in Fig. 2 time-resolved raw signal data from blanks and cocoa powdered samples are shown.

For data treatment in spICP-MS analysis, robust particle size distribution is considered when 600–1200 particles are detected. This number of particles, along with their concentration, is proposed in accordance with established guideline "The NanoDefine methods manual. Part 3, Standard operating procedures (SOPs) [40]. Considering the specifications of the ICP-MS equipment utilized, including the dwell time of 3 ms, the guideline states that no more than 2000 particles should be detected, corresponding to the particle concentration range of $2{\cdot}10^6$ to $2{\cdot}10^8$ particles L^{-1}. At this point, considering how complex food confectionary matrices are, it was difficult to reach this particle number without overlapping ionic and particulate forms. To solve this problem, many measurement replicates of each sample were performed until detecting at least 600 particles per diluted sample.

Transport efficiency was assessed with AuNPs mentioned in Section 2.4.2 and significant variations were detected between days. An average value was found to be 3.4 ± 0.2 %.

TiO_2 (nano) particles isolated from confectionary products.

For all confectionery products except sugar paste, the optimal extraction conditions (aqueous extraction with manual shaking) allowed obtaining time scans like in Fig. 3d after analyzing TiO_2 (nano)particles extracted by spICP-MS. In the case of sugar paste, a high ionic background signal of ^{48}Ti overlapped with the particulate form, regardless of the dilution rate, when the extract was analyzed by spICP-MS. This fact is also in concordance with the high total Ti content found (Table 3). To solve this problem, after the extraction of the nanoparticles, an ultrafiltration process was applied to separate the ionic and particulate form. Finally, the particulate part was resuspended and analyzed by spICP-MS obtaining time scans with a proper relation between background signal and the registered spikes.

Despite observing a calcium signal in the EDX spectrum (Fig. 1a), no Ca particles were detected during a spICP-MS screening (data not shown).

Results obtained for particle number concentration, mean size and percentage for particle under 100 nm in pure and soluble cocoa powder, sugar paste, powdered custard and sugar powder are shown in Table 6.

Although the average particle size of TiO_2 is quite similar for all the analyzed confectionary products, the size distribution varies among these products, as shown in Fig. 4. Additionally, a broad particle size distribution was observed in all cases (Fig. 4). On the other hand, TiO_2 particles under 100 nm were detected for all products. However, it is important to consider that spICP-MS detects not only individual particles but also small agglomerates. Special attention should be paid to the high percentage of nanoparticulate TiO_2 content found in soluble cocoa

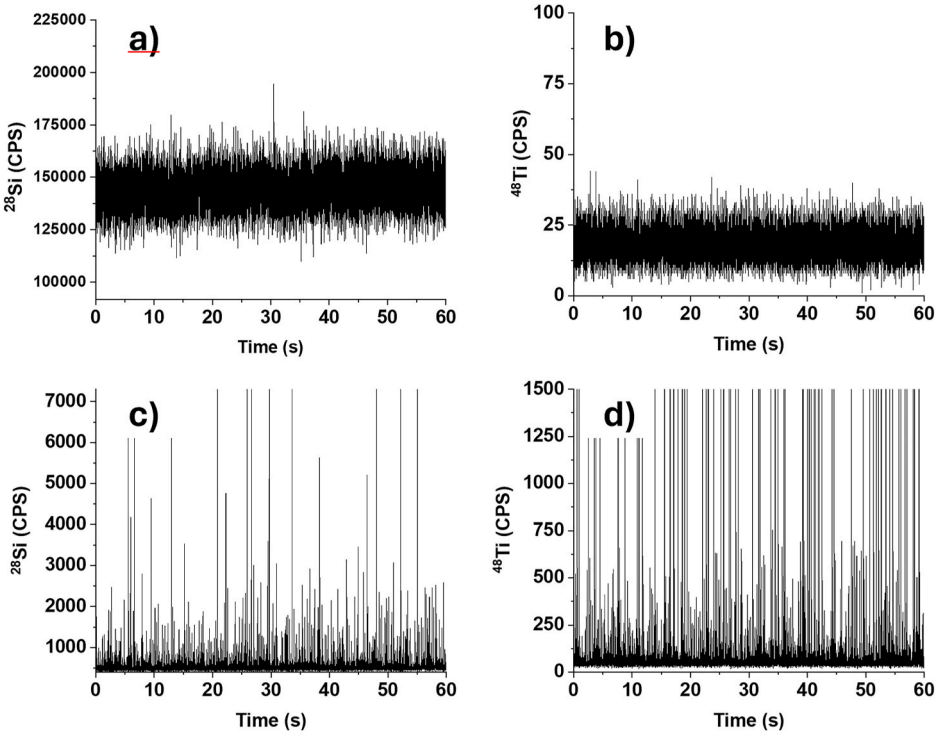

Fig. 2. Time-resolved raw signal of blank samples for ^{28}Si (a) and ^{48}Ti (b) and cocoa powder for ^{28}Si (c) and ^{48}Ti (d).

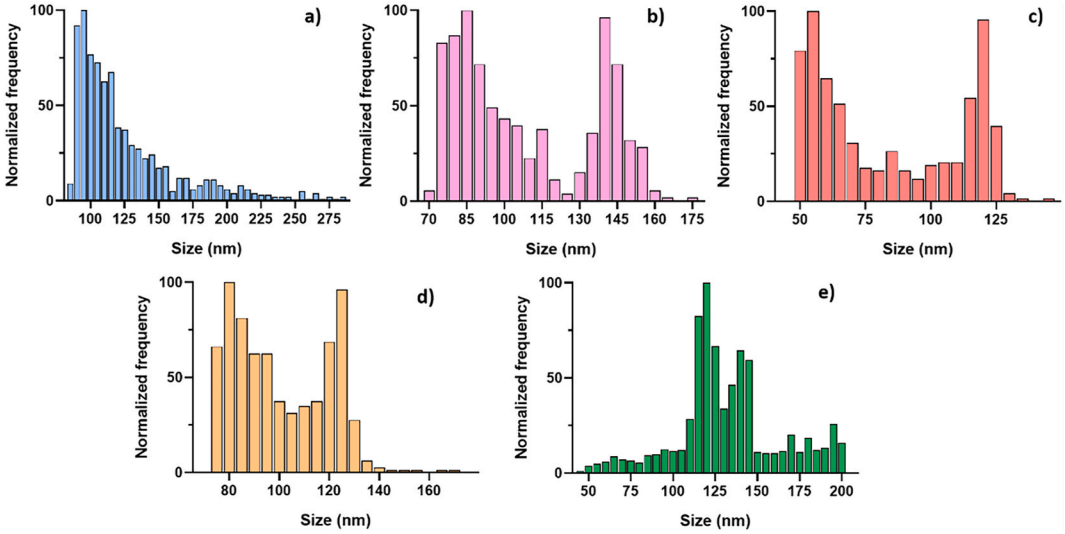

Fig. 3. Size distribution graphics for TiO_2 particles extracted from a) pure cocoa, b) powdered custard, c) sugar powder, d) soluble cocoa and e) sugar paste using water and manual shaking as optimal extraction conditions.

Table 6
TiO_2 and SiO_2 particle mean size, percentage under 100 nm and concentration in confectionary products under aqueous extraction with manual shaking.

	TiO_2 particles			SiO_2 particles	
	Mean size (nm)	% <100 nm	[TiO_2] (part g^{-1})	Mean size (nm)	[SiO_2] (part g^{-1})
Pure cocoa	126 ± 40	30 ± 4	$(2.6 \pm 0.2)\cdot10^8$	269 ± 27	$(8.2 \pm 0.5)\cdot10^8$
Soluble cocoa	125 ± 65	48 ± 6	$(1.7 \pm 0.1)\cdot10^8$	271 ± 21	$(1.1 \pm 0.1)\cdot10^8$
Sugar paste	138 ± 48[a]	9 ± 2[a]	$(8.7 \pm 0.2)\cdot10^{6}$[a]	271 ± 15	$(5.5 \pm 0.1)\cdot10^8$
Powdered custard	147 ± 72	36 ± 7	$(5 \pm 1)\cdot10^6$	278 ± 36	$(5.9 \pm 0.4)\cdot10^8$
Sugar powder	122 ± 80	47 ± 2	$(1.0 \pm 0.1)\cdot10^7$	286 ± 14	$(3.2 \pm 0.1)\cdot10^8$

All data expressed as average ± SD of three independent samples.
[a] Data obtained after ultrafiltration extraction.

and powdered sugar.

This work shows the presence of TiO_2 (nano)particles in confectionary products whose ingredient list does not include the additive E171, and in some cases the percentage of these nanoparticles is significantly high. However, detecting TiO_2 (nano)particles does not necessarily imply they originated from E171; they could potentially arise from food processing. Only the ingredient list of the sugar paste explicitly mentions the additive E171. Additionally, analysis of the size distribution depicted in Fig. 3 reveals a deviation from the typical E171 profile, characterized by a high proportion of particles in the 50–200 nm

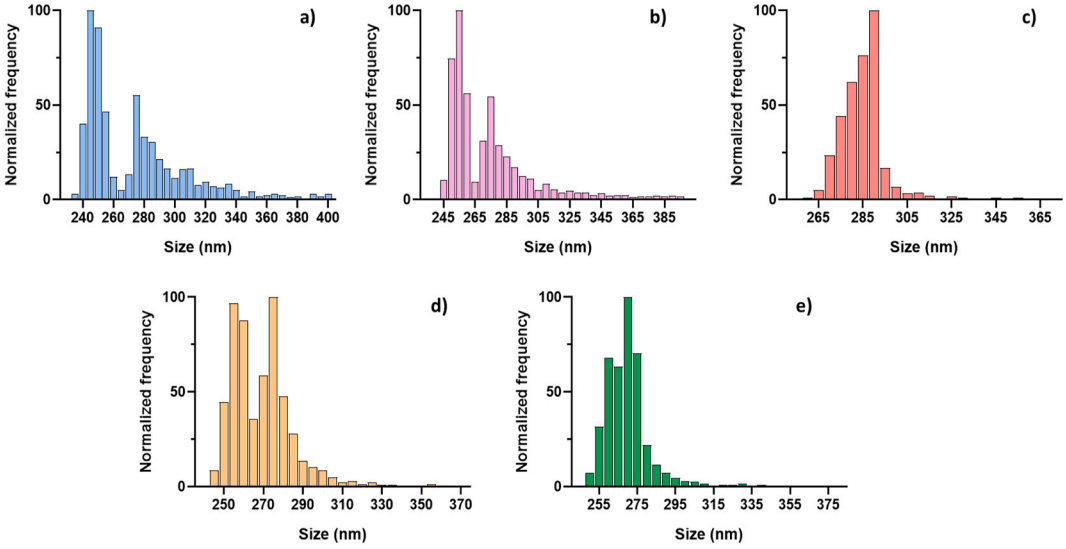

Fig. 4. Size distribution graphics for SiO_2 particles extracted from a) pure cocoa, b) powdered custard, c) sugar powder, d) soluble cocoa and e) sugar paste using water and manual shaking as optimal extraction conditions.

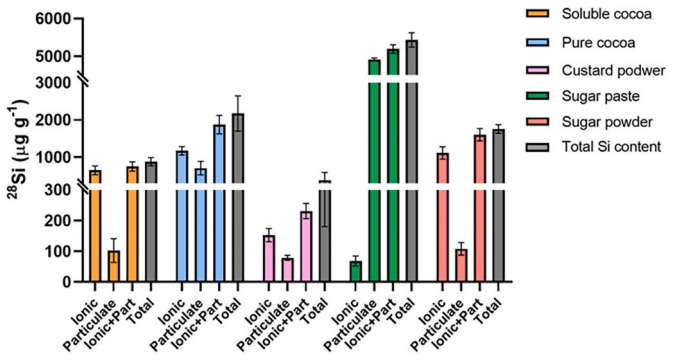

Fig. 5. Mass balance for ^{28}Si in the different confectionary products.

range [6]. This deviation could be explained to a different origin of the nanoparticles of TiO_2, justifying the absence of E171 in the ingredient list. The distinctive size distribution of E171 is apparent for the sugar paste, which is the only food sample where the additive was listed as an ingredient.

SiO_2 (nano)particles isolated from confectionary products.

Like the TiO_2 (nano)particles found, SiO_2 particles also presented a similar mean size in all confectionery products (Table 6), even though their size distributions (Fig. 4) were different. As shown in Fig. 4, SiO_2 particles were detected, and although they may not originate from the food additive E551, fall within a similar size range to that found by Ref. [41] when analyzing decorative products in confectionery.

However, the detection of SiO_2 particles was limited by the elevated LOD_{size} (142 nm). It is known that E551 occurs as small constituent particles (<20 nm), which form small aggregates, and the constituent particles and the aggregates could form agglomerates. Thus, it is likely that the sizes of these agglomerates, expressed as equivalent spherical diameter, were the one which have actually being determined. This limitation could impact both the particle concentration in the sample and the determination of the percentage of SiO_2 particles below 100 nm. Therefore, to verify that all SiO_2 particles were being analyzed, a mass balance (Fig. 5) was performed to ensure ^{28}Si was being properly measured and no over- or underestimates were being made. As it can be seen in Fig. 5, the total Si content measured by ICP-MS for each of the confectionery products is comparable with the content resulting after considering the sum of the ionic form and the particulate form concentration measured by spICP-MS for the same products. The data revealed that in all cases, most of the Si content was present in ionic form or as particles below the LOD_{size}: 86 ± 8 % for soluble cocoa, 63 ± 7 % for pure cocoa, 66 ± 10 % for powdered custard, 99.9 ± 0.3 % for sugar paste, and 65 ± 8 % for sugar powder. Therefore, In the case of SiO_2 particles, it had to be assumed that particles under LOD_{size} were being treated as ionic content, and this assumption is supported by the mass balance.

Giménez-Ingalaturre et al., [42]. proposed that for those cases where the lower limit of the particle size distribution is close to the LOD_{size} (30 nm in Milli-Q water), small particles are not being recorded. Due to the high value of the SiO_2 LOD_{size}, and in view of the size distribution plots registered in Fig. 4, the conclusions obtained in Giménez-Ingalaturre et al. [42], work may be considered. The particle size distribution graphs are biased, because of the high background present in the samples that could not be eliminated by varying the dilution, and therefore preventing the quantification of small particles. For this reason, the percentages of ionic and particulate content should be regarded as semi-quantitative data since there may be SiO_2 particles below the LOD_{size}, potentially underestimating the particulate content in such cases.

4. Conclusions

In this study, a protocol has been optimized that enables the simultaneous extraction of TiO_2 and SiO_2 (nano)particles while preserving their morphological conditions. TEM and spICP-MS analysis revealed that the use of enzymes, as well as the application of ultrasonics (both bath and probe) in the extraction processes, did not improve the quantity of extracted particles. In the case of enzymes, their use as extractant agents complicated the subsequent analysis of the extracts by spICP-MS. Therefore, water as extractant agent and manual shaking were selected as optimal extraction conditions to have a simultaneous TiO_2 and SiO_2 (nano)particles extraction procedure. The selected sample treatment offers a fast, cost-effective and environmentally friendly protocol for extracting SiO_2 and TiO_2 particles in such samples.

(Nano)particles of TiO_2 and SiO_2 have been identified under the optimal extraction conditions through TEM and spICP-MS in all analyzed confectionary products, despite the corresponding additives, E171 and E551, were not indicated on the label (except for sugar paste). The TiO_2 (nano)particles analyzed by spICP-MS showed an average percentage of nanoparticles of 30 %, being the percentage under 100 nm very close to 50 % in the case of soluble cocoa and sugar powder. Therefore, even with the ban on using the E171 additive in Europe, ongoing surveillance is essential to detect the presence of nanoscale TiO_2 in these foods, and in those whose labeling does not include it but are suspected to contain it. Results for SiO_2 particles should not be considered quantitative because the high background did not allow to determine particles bellow 100 nm. Therefore, these results highlight the importance of developing extraction methods that do not affect the physicochemical properties of (nano)particles when extracted from complex matrices such as confectionary products. In this way, spICP-MS screening analyses can be performed to monitor the potential presence of TiO_2 and SiO_2 (nano)particles since they might appear in the final products in case of confectionery even when they are not included in the ingredient list or banned by the authorities. This fact is of key importance for ensuring food safety, regulatory adherence, and transparent consumer information.

CRediT authorship contribution statement

Elena Espada-Bernabé: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Beatriz Gómez-Gómez:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Gustavo Moreno-Martín:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Yolanda Madrid:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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through a research support contract (CT4/21-CT5/21).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2024.343058.

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