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Prospects and difficulties in TiO_2 nanoparticles analysis in cosmetic and food products using asymmetrical flow field-flow fractionation hyphenated to inductively coupled plasma mass spectrometry

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ABSTRACT

In this work, we proposed an analytical approach based on asymmetrical flow field-flow fractionation combined to an inductively coupled plasma mass spectrometry (AsFlFFF–ICP-MS) for rutile titanium dioxide nanoparticles (TiO_2NPs) characterization and quantification in cosmetic and food products. AsFlFFF–ICP-MS separation of TiO_2NPs was performed using 0.2% (w/v) SDS, 6% (v/v) methanol at pH 8.7 as the carrier solution. Two problems were addressed during TiO_2NPs analysis by AsFlFFF–ICP-MS: size distribution determination and element quantification of the NPs. Two approaches were used for size determination: size calibration using polystyrene latex standards of known sizes and transmission electron microscopy (TEM). A method based on focused sonication for preparing NPs dispersions followed by an on-line external calibration strategy based on AsFlFFF–ICP-MS, using rutile TiO_2NPs as standards is presented here for the first time. The developed method suppressed non-specific interactions between NPs and membrane, and overcame possible erroneous results obtained when quantification is performed by using ionic Ti solutions. The applicability of the quantification method was tested on cosmetic products (moisturizing cream). Regarding validation, at the 95% confidence level, no significant differences were detected between titanium concentrations in the moisturizing cream prior sample mineralization (3865 ± 139 mg Ti/kg sample), by FIA–ICP-MS analysis prior NPs extraction (3770 ± 24 mg Ti/kg sample), and after using the optimized on-line calibration approach (3699 ± 145 mg Ti/kg sample). Besides the high Ti content found in the studied food products (sugar glass and coffee cream), TiO_2NPs were not detected.

1. Introduction

The presence of nanomaterials (NMs) is becoming common in a wide range of products and sectors including medicine, cosmetics, clothing, engineering, electronics, and food. Titanium dioxide nanoparticles (TiO_2NPs) are frequently used as additives and glidants ("anti-caking agents") in the food industry, and are also included in sunscreens and cosmetics products [1–3]. The TiO_2NPs included in sunscreens act as a shield against UV radiation. Many toxicological studies have been performed and several in vitro and in vivo toxicity tests have showed that TiO_2NPs do not penetrate the human skin [4,5]. However, there is some controversy on the mechanisms involved in TiO_2-induced genotoxicity and carcinogenicity [6–8].

Knowledge on size, shape, surface area, aggregation state, charge, chemistry, and reactivity of NPs is essential when evaluating their potential toxicity and behavior. To obtain correct information on the physical and chemical properties of NPs, reliable quantitative methods of analysis are needed. A wide range of analytical techniques such as microscopy, chromatography, centrifugation and filtration, spectroscopic, and other related methods have been used for various purposes: characterization of particle size, morphology and aggregation state [9], chemical characterization [10], and surface chemical analysis [11]. Electron microscopy is often used in NPs studies, since it allows identifying the presence of these particles, providing useful information on size distribution and other measurable properties [5,12]. However, electron microscopy is not always available and the average particle size depends on a limited number of measured particles. Currently, field-flow fractionation (FFF) is a suitable technique for size separation of natural and inorganic NPs. The two most commonly used FFF subtechniques for TiO_2 NPs analysis are sedimentation (SedFFF) and the asymmetrical flow field-flow fractionation (AsFlFFF) [13–15]. Multi-angle laser light scattering (MALLS) and UV detectors are the most common systems used in combination with FFF, but this separation technique can be coupled to sensitive and multi-element detectors such as mass spectrometers (ICP-MS) [16]. The main advantage of FFF–ICP-MS combination is not only the multiple element detection capability, but also the higher sensitivity compared to UV detectors, reaching detection limits in the range of ng/L.

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Although AsFlFFF–ICP-MS is considered a promising approach, it has some poorly understood instrumental limitations; the need to re-equilibrate the membrane in order to avoid the accumulation of NPs [17], or the limited mass sensitivity due to the high sample dilution occurring during migration in the channel up to detector. Furthermore, development of analytical methodologies requires special attention during sample preparation and optimization of the run conditions, which have to be focused on certain type of particles and matrices [18].

The aim of this work is to assess the possibilities and difficulties of AsFlFFF–ICP-MS in the characterization and elemental quantification of TiO_2NPs in cosmetic and food products available in the market. The experimental parameters that affect NPs separation and elemental quantification by AsFlFFF–UV/ICP-MS are here optimized and discussed. In addition, size distribution results from AsFlFFF were validated through transmission electron microscopy (TEM). For the first time, a highly promising approach for quantitative determination of TiO_2NPs without the need of additional strategies and avoiding the use of calibration standards of different nature is presented in this work.

2. Materials and methods

2.1. Chemicals and samples

Standard TiO_2NPs dispersions were prepared using rutile TiO_2 nanopowder (<100 nm particle size) from Sigma (St. Louis, USA). According to the manufacturer, nanopowder may contain up to 5 wt% silicon dioxide as surface coating and a small amount of anatase (<25 nm particle size). Additional information regarding to specific surface area (130–190 m^2/g) and density (4.17 g/mL at 25 °C) is provided. Our analyses by TEM showed these NPs highly aggregated making the determination of the nanoparticle size a difficult issue. Ti elemental standard solution of 1000 mg/L used to prepare ICP-MS standard solutions was purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (98.5%) from Sigma (St. Louis, USA) and methanol (HPLC grade) from Scharlau (Walkerburn, Scotland) were needed to prepare the carrier solution for the AsFlFFF system. Sodium hydroxide from Panreac (Barcelona, Spain) was used to adjust the pH of the carrier solution to 8.7. Ultrapure Milli-Q water (Millipore, MA, USA) was used to prepare standard and carrier solutions. Polystyrene latex standards with diameters of 22, 54, and 100 nm were purchased from Postnova Analytik (Landsberg, Germany). Nitric acid (HNO_3) (65%) and hydrofluoric acid (HF) (47–51%) from Merck (Darmstadt, Germany), as well as hydrogen peroxide (H_2O_2) (35%) and boric acid (H_3BO_3) from Panreac (Barcelona, Spain) were used to mineralize the samples. Hexane for defatting was purchased from Scharlau (Walkerburn, Scotland).

The foodstuffs (sugar glass and coffee cream) and SPF 10 moisturizing cream were purchased in the market.

2.2. Preparation of standard TiO_2NPs dispersions

A standard dispersion of TiO_2NPs at 1000 mg/L was prepared by suspending the corresponding amount of rutile TiO_2 nanopowder in ultrapure water. To prevent particle aggregation the dispersion was sonicated in an ultrasonic bath for 10 min. For AsFlFFF–ICP-MS analysis, working standards dispersions were daily prepared using a 10-fold dilution of 1000 mg/L standard in the carrier solution and then tip sonicated for 2 min.

2.3. Instrumentation

An AF2000 AsFlFFF system (Postnova Analytik, Landsberg, Germany), equipped with a regenerated cellulose membrane of 10 kDa molecular weight cut-off and a spacer of 350 μm, was used in this study. A 200 μL injection loop was used for sample injection into the AsFlFFF system via a Rheodyne valvule. The AsFlFFF channel was connected on-line to an Agilent Infinity 1260 UV detector (Agilent, CA, USA) and to a 7700× ICP-MS instrument (Agilent Technologies, Santa Clara, CA, USA), equipped with a cooled Scott-type spray chamber and a slurry nebulizer. The optimized AsFlFFF settings, flows used for separating the NPs, and ICP-MS operating conditions are detailed in Table 1.

For FIA–ICP-MS measurements, a PU-2089 LC pump (JASCO, Tokyo, Japan), fitted with a six-port injection valve (Model 7725i, Rheodyne, Rohner Park, CA, USA) with a 100 μL injection loop and peek tube, was needed for off-line ICP-MS analysis. The outlet of the peek tube (inner diameter = 0.13 mm) was directly connected to the slurry nebulizer of the ICP-MS system.

A 1000 W MSP microwave sample preparation system (CEM, Matthews, NC, USA) equipped with temperature and pressure feedback controls and 12 high-pressure vessels of 100 mL inner volume, operating at 1600 W, was employed for the digestion processes.

For stabilizing NPs dispersions, a Vibra cell VCx130 ultrasonic processor (Connecticut, USA) equipped with a titanium 2-mm-diameter microtip and fitted with a high-frequency generator of 130 W at frequency of 20 kHz was used.

An Eppendorf Centrifuge 5804 R (Hamburg, Germany) was used to separate the hexane fraction from the solid residue after the extraction procedure.

An Eppendorf Vacufuge plus concentrator (Eppendorf AG, Hamburg) was used to evaporate the liquid solvent after collecting the fraction from the AsFlFFF system in order to concentrate TiO_2NPs for further analysis.

Transmission electron microscopy (TEM) analyses were performed using a JEM 2000FX microscope (JEOL, Tokyo, Japan) at 200 kV.

For accurate characterization of TiO_2NPs, fractions containing NPs were collected from the AsFlFFF system, concentrated, and analyzed by TEM. Samples were prepared by placing droplets of dispersion, previously tip sonicated for 2 min, onto a holey carbon film on copper grids.

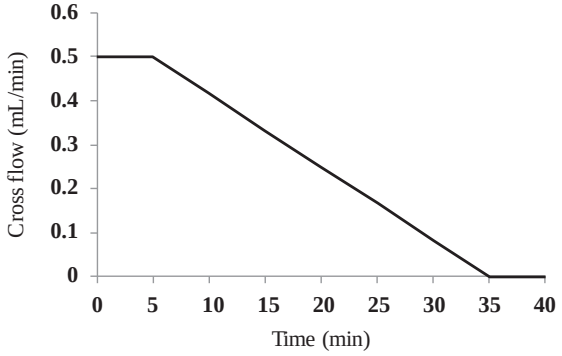
2.4. Microwave acid digestion of moisturizing cream and food samples

Approximately 0.1 g of sample was placed into a double-wallet advanced composite vessel (ACV) for microwave-assisted digestion. The mineralization process was performed following the procedure of Contado et al. with some modifications [14]. First, 6 mL of HNO_3 (65%), 3 mL of concentrated HF (47–51%), and 1 mL of H_2O_2 (35%) were added to the sample. Then, the vessels were sealed and subjected to the following digestion program: step 1, a 15 min linear ramp from 0 to 210 °C; and step 2, holding the temperature at 210 °C for 10 min. Samples were cooled and then, 1.5 g of H_3BO_3 were added to each vessel to complex the residual hydrofluoric acid or redissolve insoluble fluorides formed. In this step samples were mineralized by applying the following program: an initial 10 min linear ramp from 0 to 170 °C, followed by holding the temperature at 170 °C for 10 min. The digested extracts were transferred to volumetric flasks and diluted to 100 mL with ultrapure water. All digestions were performed in triplicate.

2.5. TiO_2NPs AsFlFFF–ICP-MS quantification in extracts from moisturizing cream

Two different quantification methods were applied to the TiO_2NPs extracted by applying the procedure of Nischwitz et al. [15]. Briefly, 0.1 g of sample was weighed into a Falcon tube

Table 1
Instrumental parameters of AsFlFFF system.

AsFlFFF operating parameters	
Spacer height	350 μm
Membrane type	Regenerated cellulose (RC) with 10 kDa MWCO
Injection loop	200 μL
Carrier liquid	0.2% (w/v) SDS, 6% (v/v) MeOH at pH 8.7
Injection flow	0.2 mL/min
Focus flow	1.3 mL/min
Cross flow	0.5 mL/min
Detector flow	1 mL/min
Injection time	4 min
Elution program	
UV detection of TiO_2 NPs	λ=270 nm
UV detection of polystyrene latex standards	λ=280 nm
ICP-MS operating conditions and data acquisition parameters	
Forward power	1550 W
Plasma gas flow rate	15 L/min
Carrier gas flow rate	0.98 L/min
Nebulizer gas flow rate	0.83 L/min
Dilution gas	0.3 mL/min
Collision gas	Helium
Collision gas flow rate	4.3 mL/min
Nebulizer	Slurry
Spray chamber	Cooled Scott type spray chamber
Isotope monitored	^{47}Ti, ^{49}Ti and ^{103}Rh
Dwell time per point	500 ms

(50 mL) and 10 mL of hexane were added. The tube was vigorously shaken and sonicated using an ultrasonic bath for 1 min. The mixture was allowed to settle for 1 h and then centrifuged at 3000 rpm for 5 min. The organic solvent was removed and 20 mL of ultrapure water were added to the solid residue. The tube was sonicated for 30 min in a water bath. All extractions were performed in triplicate.

In the case of foodstuff, samples were weighed and directly sonicated under same conditions.

2.5.1. On-line AsFlFFF–ICP-MS calibration

External calibration curve was prepared by using rutile TiO_2NPs as standard. Different concentrations were prepared by diluting a working standard dispersion of 100 mg TiO_2NPs/L in the appropriate volume of carrier solution. Before injecting into the AsFlFFF–ICP-MS system, the dispersions employed in external calibration were also sonicated for 2 min.

2.5.2. Off-line AsFlFFF–ICP-MS calibration

Sample extracts were injected into the AsFlFFF system and the fractions corresponding to UV signal of NPs were collected from the AsFlFFF channel. Ti content in each fraction was quantified by FIA–ICP-MS using a standard addition method following two different approaches: (a) by spiking several aliquots of each fraction with increasing concentrations of Ti elemental standard solution, and (b) by spiking several aliquots of each fraction with increasing concentrations of rutile NPs TiO_2NP standard dispersion.

In both approaches a 50 μg/L of ^{103}Rh solution was added into sample extracts as the internal standard. Data were obtained in Time Resolved Analysis (TRA) mode and signals were integrated as peak area.

3. Results and discussion

3.1. Optimization of the parameters affecting TiO_2NPs rutile standard separation by AsFlFFF

The separation of NPs by AsFlFFF is directly affected by experimental and instrumental parameters. Concerning this latter, the type of membrane and spacer dimensions play an important role in NPs separations. Out of the available membranes, regenerated cellulose (RC) is frequently used in analyses of inorganic NPs. This type of membrane is less hydrophobic than polyethersulfone (PES) and polyvinylidenedifluoride (PVDF) which could enhance NPs recovery. Based on previous studies reported by other authors, in this work RC membrane and a 350 μm thick spacer were chosen [14,15,19,20].

Regarding carrier composition, parameters such as ionic strength, surfactant agent, and the pH of the carrier solution can affect NP–NP interactions or NPs-membrane interactions [17,21,22]. It has been reported that at low ionic strength conditions TiO_2 aggregation

decreases [14]. Thus, preliminary experiments were carried out in presence of 0.03, 0.003, and 0.001 mol/L $NaNO_3$. The increase of the ionic strength led to irreversibly adsorption of large particles onto the membrane (data not shown), and therefore losses in the channel and low recoveries, which was in line with results obtained by other authors [23]. For these reasons, the use of electrolytes was discarded in further experiments. Carriers with pH values between 7 and 9 are often used in AsFlFFF [20,24,25]. Hence, considering that TiO_2NPs in rutile phase are stabilized in alkaline solution [26], we decided to use a carrier solution at pH 8.7. We also observed that an improvement in NPs separations was obtained through the AsFlFFF system with the addition of SDS in the carrier liquid in comparison with water. Three SDS concentrations (0.01%, 0.1%, and 0.2% (w/v)) at basic pH were tested using different separation programs (by applying different cross flow rates and elution modes). By using the lowest SDS concentration, the most of the injected NPs eluted at 0 mL/min cross flow (Fig. S1a). The increase of SDS concentration prevented non-specific NPs-membrane interactions and provided a better rutile TiO_2NPs standard fractionation. Marked variations in UV profiles were obtained when the carrier solution contained 0.2% (w/v) SDS (Fig. S1c). This concentration was used for subsequent experiments. Besides the positive effects of surfactant in AsFlFFF analysis, SDS can affect the nebulization efficiency of NPs when the system is coupled to ICP-MS. For preventing this negative effect, Schmidt et al. added 3% (v/v) methanol to the carrier in order to enhance and stabilize the ionization efficiency of Au NPs [16]. Additionally, it is know that the use of organic solvents may prevent TiO_2NPs aggregation and enhance the resolution of the fractograms [15,27]. Because of this we decided to use methanol in carrier solution (0.2% (w/v) SDS and 3% (v/v) methanol at pH 8.7). Although the mechanism by which methanol controls the aggregation of NPs is not fully understood, it has been reported that this organic solvent may form a layer on the particle surface improving the dispersion of NPs in aqueous solutions [28].

Cross flow is one of the main parameters controlling the distribution of particles near the membrane and consequently their separation [29,30]. We optimized the cross flow at a constant and gradient elution mode. At high cross flow rate (2 mL/min), interaction of the NPs with the membrane increased and most of the sample eluted when the cross flow was at 0 mL/min, as TiO_2NPs that were non-specifically adsorbed to the membrane were released when the cross flow was interrupted (Fig. S2a). The tendency of TiO_2NPs to form aggregates larger than 100 nm led us use cross flow rates below 2 mL/min. A cross flow of 1 mL/min did not improve the fractionation since a high intensity peak at 0 mL/min cross flow was observed (Fig. S2b). On the contrary, the behavior of the rutile TiO_2NPs standard using a cross flow of 0.5 mL/min and a linear decay of the flow rate in 10 min was promising, but required further optimization (Fig. S2c.1). Considering that the gradient time was not enough to achieve a complete NPs recovery in the elution step, longer gradient times were tested (Fig. S2c.2). On this way, we can achieve that a decrease of the field applied occurs at a lower rate allowing more time for the particles adhered to the membrane are released to the channel when the field decreases. By applying a 30 min gradient time, the peak corresponding to residual NPs disappeared and the NPs eluted within a range of 15–32 min (Fig. S2c.3). Thus, a 0.5 mL/min rate was chosen as the optimal cross flow, which was in line with the value reported by other authors [29].

Finally, the influence of injection/focusing time on the separation of TiO_2 NPs was evaluated. During injection time, NPs are focused in a narrow zone on membrane under focus flow applied. Previous tests performed using an injection time of 4 min and after the AsFlFFF –ICP-MS coupling corroborated that the peak appeared at 5 min corresponded to void peak containing TiO_2 NPs, which could be due to presence of a small particles fraction in the standard dispersion, or a possible "memory effect" due to a release of NPs during carrier injection. In order to evaluate if shorter or higher injection time affect to the presence of TiO_2 NPs at the void peak, the injection time was decreased (from 4 min to 1 min). The recovery of NPs became worse and the fraction of eluted NPs increased in the void peak. On the other hand, longer injection times only proved a displacement of retention times (data not shown). Therefore an injection time of 4 min were selected for further experiments.

3.2. Recovery and reproducibility problems of AsFlFFF rutile TiO_2NPs fractionation

Low recovery values could be attributed to undesirable membrane–particle interactions. Adsorbed NPs can modify the membrane surface charge, preventing further adsorption [16]. In our study, about 5 consecutive injections to stabilize the membrane before sample fractionation were enough.

The ratio of peak areas of the fractograms and the amount of sample injected was used as a first assessment of recovery. Initially, we had serious problems in terms of reproducibility and recovery (Fig. S3). The effect of methanol concentration change from 3% (v/v) to 6% (v/v) to avoid sample loss and/or adhesion of NPs to surfaces of the devices, including the bottom membrane, was checked. Results showed that when 6% (v/v) methanol was used the ratio of peak areas were proportional to the ratio of the amount of TiO_2NPs injected (about $r=2$) (Fig. S3b). Considering this, we concluded that a 0.2% (w/v) SDS and 6% (v/v) methanol at pH 8.7 was the optimal composition for the carrier solution.

3.3. AsFlFFF–ICP-MS fractionation of the rutile TiO_2NPs. Confirmation of size distribution by transmission electron microscopy

Using the previously optimized conditions, two peaks were obtained in the fractograms after the injection of the rutile TiO_2NPs dispersion and UV/ICP-MS detection (Fig. 1a). The first peak appeared at 5–6.5 min and could correspond on one hand to a residue of the void peak or to a memory effect due to the deposition of NPs in the focus zone, and on the other hand to low size NPs present in the dispersion. The second peak appeared at 12–32 min, it was quite broad and could correspond to highly aggregated TiO_2NPs [13–15]. To date, obtaining adequate information on NPs sizes is still an important issue in AsFlFFF analyses. It is known that the separation of NPs in an AsFlFFF channel occurs according to their diffusion coefficient, which can in turn be related to hydrodynamic particle size or molecular weight. Different strategies have been proposed to determine the hydrodynamic diameter of fractionated particles, such as the FFF theory or the size calibration methods using standards of known size. Both approaches entail that size fractionation is only dependent on the size of the component but independent of its chemistry. However, erroneous information may still be obtained since these calibration methods do not take into account elution time changes due to specific NP-membrane interactions, different behavior between the calibration particles and the analyte, or the physical and chemical properties of the NPs leading to different diffusion coefficients [17,31]. Moreover, the application of FFF theory equations may give us erroneous diffusion coefficients due to the anisotropy of the nanoparticles or their aggregates.

We constructed a size calibration using polystyrene latex standards of three known sizes (22, 54, and 100 nm) and estimated that the particles identified in the first peak had a hydrodynamic diameter below 22 nm, whereas aggregated particles (higher than 100 nm in size), showing a wide dispersion, eluted at 12–32 min (Fig. 1a). Sometimes, elution times cannot be associated with the real size of the NPs, e.g., when NPs are

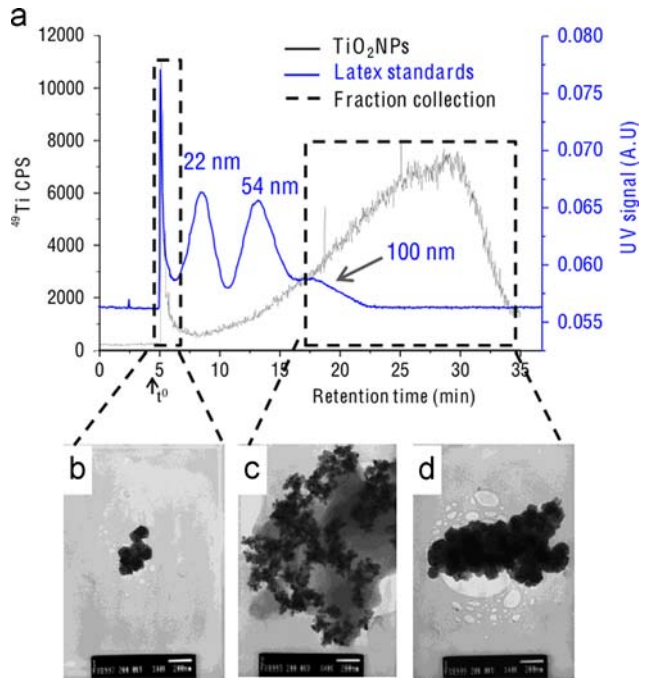


Fig. 1. Overlaid fractograms corresponding to (a) the separation of a mixture of polystyrene latex standards and rutile TiO_2NPs standard analysis by AsFlFFF–UV and AsFlFFF–ICP-MS, respectively. TEM images of rutile TiO_2NPs standard aggregates found in fractions collected from the AsFlFFF system: (c) high magnification images of a fraction corresponding to the broad peak shows numerous TiO_2NPs aggregates of around 200 nm, and (d) larger aggregates whose size reached 1 μm. (b) TEM images of the narrow peak ("void peak") shows a less pronounced aggregation state. The scale bar represents 200 nm and the magnification of the images is 40k. The black arrow (t^0) indicates the void peak.

aggregated or when other components of the sample interact with the membrane, slowing down or accelerating the elution time of the particles.

Due to the NPs-membrane and latex standards-membrane interactions may be different, the use of an independent method such as electron microscopy is key to determine the primary particle size and morphology of dry NPs. Fractions corresponding to Peak 1 and Peak 2 were collected and evaporated prior TEM analysis. Comparison of both methods, particle size obtained by TEM with the average size calculated using latex standards, showed the following: TEM images corresponding to the narrow Peak 1 revealed the presence of TiO_2 aggregates of lower size (Fig. 1b). However, NPs size of around 80 nm indicated that the elution time did not correspond to the expected size. These results reveal that NPs that eluted earlier, identified in the void peak, correspond to NPs with a smaller size in comparison with those that eluted later which is in concordance with the AsFlFFF separation concept. Aggregates of around 200 nm (TEM) fit with the broad size distribution (second peak) observed in AsFlFFF–ICP-MS (Fig. 1c). Furthermore, rests of surfactant on the surface of the aggregates could be observed, suggesting SDS may adsorb on the surface of the particles, preventing them from interacting [32,33]. It is important to note that TEM images obtained from Peak 2 revealed aggregates larger than 200 nm in size, reaching 1 μm approximately (Fig. 1d). These findings could indicate that the use of latex standards for calibrating rutile TiO_2NPs size could lead to erroneous results in size distribution assignment, mainly due to the lack of spherical TiO_2NPs and their high aggregation state [34]. However to corroborate this hypothesis the retention time of standards of higher sizes such as 500 nm and 900 nm should be checked and related to our NPs.

3.4. Effect of ultrasound energy on AsFlFFF recovery

As we have commented before, in FFF analysis a combination of parameters has to be optimized in order to achieve good reproducibility and recovery. Separation of NPs is not only affected by instrumental parameters such as carrier composition or cross flow rate. Preparation of NPs dispersions for AsFlFFF analysis is a critical step, since solvent changes, pH, or even dilutions may affect their agglomeration and behavior. Although TiO_2NPs are not able to separate (themselves) into individual particles [34], it has been shown that ultrasound energy slightly reduces agglomeration. However, it is important to note that the optimization of ultrasound methodology is directly related to sample treatment, so it is convenient to distinguish this parameter from other instrumental and experimental parameters such as cross flow, focussing/injection time or carrier solution composition.

In this study, the effect of the type of ultrasound (ultrasonic bath vs. ultrasonic probe) on aggregation and the sonication time were optimized. The fractogram profiles obtained with both sonication techniques were quite similar but recovery using the ultrasonic probe (at 40% ultrasound amplitude for 2 min) increased in comparison to the ultrasonic bath (Fig. 2).

3.5. AsFlFFF–ICP-MS quantification of TiO_2NPs: development of an external calibration approach

There are different quantification approaches and the most commonly used are: off-line quantification of fractions collected from the AsFlFFF system followed by ICP-MS, detection after acid digestion, and on-line AsFlFFF–ICP-MS analysis using ionic standards added to the flow coming from AsFlFFF system using a mixing-T [14–16]. The use of external calibration approaches (with standards of the same chemical nature as the analytes) in AsFlFFF–ICP-MS analyses provides simultaneous information on NP quantity and size, without the need of alternative strategies. Moreover, possible differences in ionization and transport efficiencies between NPs and their corresponding ionic species in the mass spectrometer may be avoided to achieve a correct quantification. However, it is important to note that the surface properties of NPs standards and analyte may not be the same and the differences in NPs–NPs and NPs–membrane interactions could lead to an over- or underestimation of the recoveries. Otherwise, the lack of available certified standards for inorganic NPs is still a problem in

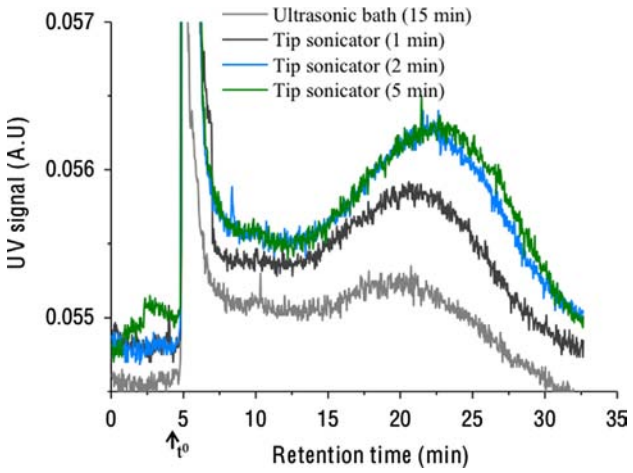


Fig. 2. AsFlFFF–UV fractograms showing the effect of ultrasound energy on the recovery of the rutile TiO_2NPs standard. The black arrow (t^0) indicates the void peak.

NPs quantification field so many times the use of ionic standards is one of the most frequent approaches.

Here, we have developed an external calibration approach using the rutile TiO_2NPs standard. It was observed that the construction of external calibration graphs was complicated due to its aggregation-dependant response. A working standard dispersion of 100 mg TiO_2NPs/L was employed for preparing the different standard dispersion injected into AsFlFFF. Two external calibration curves were generated (Fig. 3a and b). The fractograms shown in Fig. 3b were obtained by injection of a series of rutile TiO_2NPs dispersions (5, 10, 15 and 20 mg TiO_2NPs/L, corresponding to 1, 2, 3 and 4 μg TiO_2 injected, respectively) prepared in 1 mL of carrier solution and sonicated in an ultrasonic bath for 10 min. In the same way, other set of rutile TiO_2NPs dispersions (2.5, 7.5, 10 and 20 mg TiO_2NPs/L, corresponding to 0.5, 1.5, 2 and 4 μg TiO_2 injected, respectively), was used for constructing the external calibration curve shown in Fig. 3b. In this case, the AsFlFFF–ICP-MS analysis was performed after sonication using a tip sonicator for 2 min.

The fractograms did not reveal a proportional increase of ^{49}Ti signal intensity (integrated as peak area) in a concentration-dependent manner when the ultrasonic bath was used (Fig. 3a). On the contrary, when focused energy was applied, good reproducibility of fractograms, in terms of peak shape and maximum elution times, was achieved (Fig. 3b). The response obtained in a preliminary calibration approach (using ultrasonic bath sonication) was not linear (Fig. 3c); however, a successful linear plot (R^2=0.9964 and R^2=0.9952 for Peak 1 and Peak 2, respectively) and slopes with similar sensitivity were obtained in a second approach (Fig. 3d). These findings prove that tip sonication is more

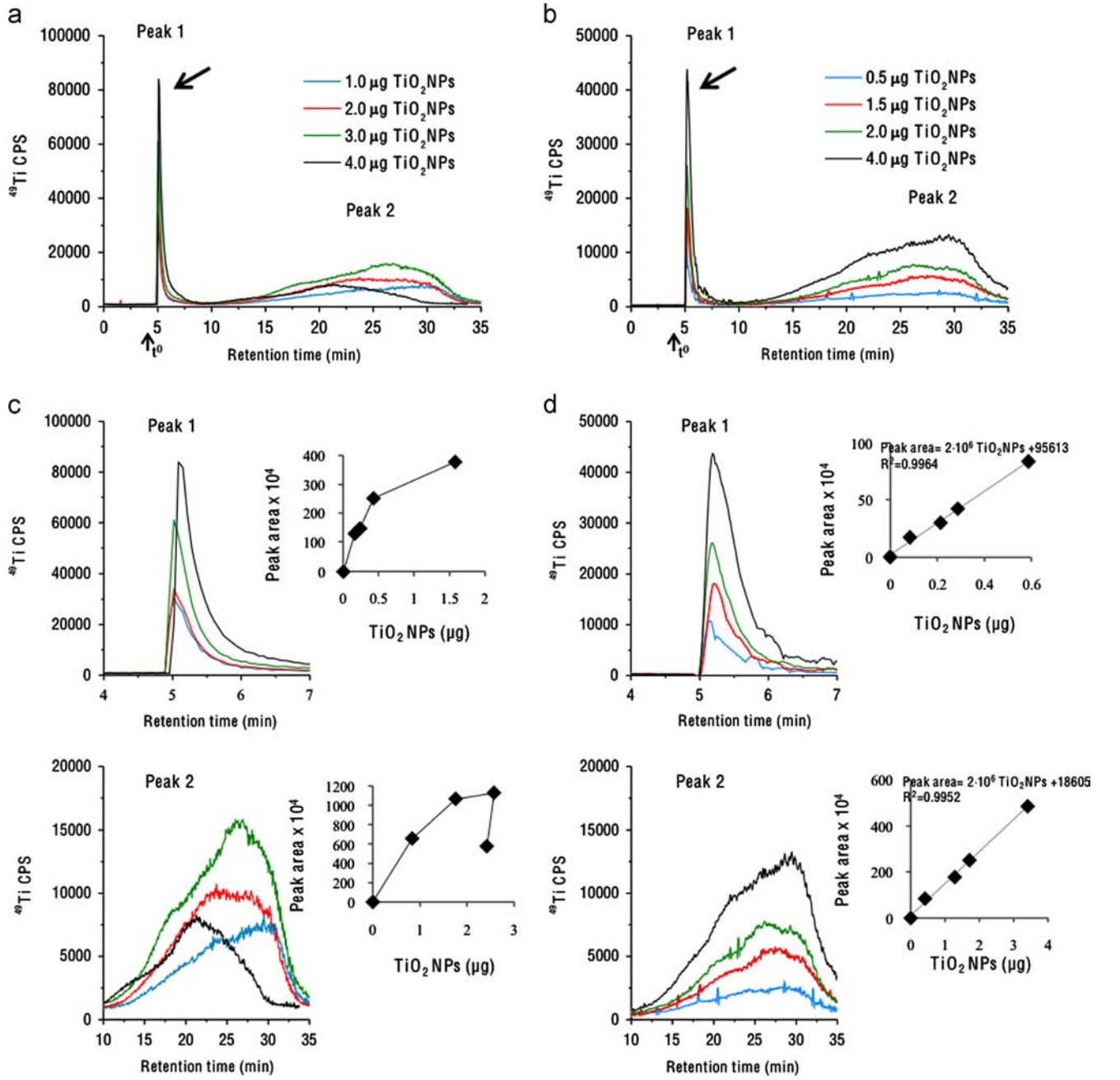


Fig. 3. AsFlFFF–ICP-MS fractograms obtained after injection of different amounts of the rutile TiO_2NPs standard. The fractograms show the external calibration approach carried out by using (a) ultrasonic bath (for 10 min) or (b) tip sonicator (for 2 min) in standard stabilization. (c and d) Peak 1 and Peak 2 magnification are shown below with their corresponding calibration curves. The black arrow (t^0) indicates the void peak.

appropriate than conventional baths for further quantification of TiO_2NPs. The adding up of Peak 1 and Peak 2 areas presumably corresponds to the whole injected NPs standard. Since areas ratio between Peak 2 and Peak 1 were similar in each calibration point, it was concluded that the amount of rutile TiO_2NPs eluted at 5 min represents 14% of the total mass, whereas the remaining titanium, corresponding to 86%, eluted at 17–32 min. In order to verify these results, fractions from Peak 1 and Peak 2 were collected, evaporated using an eppendorf vacufuge concentrator (1400 rpm, 30 °C), and subjected to acid digestion. The Ti content was determined by ICP-MS and the values obtained were the same as the percentages calculated theoretically (12.5 ± 0.2% and 87.4 ± 0.6% for Peak 1 and Peak 2, respectively). We believe our results are very promising. A suitable on-line calibration strategy using rutile TiO_2NPs standards could be of great help for the correct quantification of the TiO_2NPs present in samples, minimizing the risks of getting erroneous information, and without the need of additional strategies.

3.6. AsFlFFF–ICP-MS detection and elemental quantification of TiO_2NPs in moisturizing cream and food products

Total titanium content in moisturizing cream and food products were determined by ICP-MS following the procedures specified in Section 2. The concentration of Ti found in food and cosmetic products are shown in Table 2 (column 2).

Next, TiO_2NPs from samples were extracted following the conditions previously optimized by Nischwitz et al. [15]. Extraction efficiency was established by comparing the amount of titanium obtained by acid digestion and extraction protocol from moisturizing cream. One difficulty found when analyzing NPs by ICP-based techniques was the differences in nebulization efficiencies between NPs and the ionic standard solutions. With the aim of evaluating the extent of this effect, two external calibration approaches based on measuring the areas of the peaks obtained by FIA–ICP-MS with rutile TiO_2NPs (Method 1) and ionic Ti (Method 2) were performed. The sensitivity of Method 2 was almost three times higher than for the Method 1. This fact corroborated the differences in the efficiency of nebulization for both species. The Ti concentrations found in the aqueous extracts of the moisturizing cream using Method 1 (Table 2, column 3) was very similar to the Ti concentrations previously quantified after acid digestion (Table 2, column 2). These results prove that the Ti in the sample is present as TiO_2NPs (3770 ± 24 mg Ti/kg sample).

Finally, food samples were treated under similar conditions, but a negligible Ti signal was detected. In addition, TEM analysis did not reveal presence of TiO_2 in the food extracts. Our results could support the fact that although TiO_2 is commonly used as anti-caking agent and food additive (E171), this compound is not present in NPs form. Currently, there are not evidences of TiO_2 NPs in foodstuff.

Fig. 4 shows the particle size distribution pattern of moisturizing cream extracts. Recovery percentages were calculated by comparing the Ti concentration in the mineralized moisturizing cream sample (SPF 15) with Ti concentration in the mineralized fraction collected from the AsFlFFF system under the experimental conditions used in this study (8–22 min). An average recovery of 85 ± 4% was obtained, below 100% because the first fraction (5–6.5 min) was not collected. The recovery reported for moisturizing cream following this procedure was similar to the recovery value obtained in the fraction collected when sample was eluted after focusing but without application of the cross flow (92 ± 1.2% for Peak 2). Furthermore, it was noted that the Ti percentage found by both procedures was according to the Ti ratio quantified in Peak 1 and Peak 2 after analysis of 10 and 20 mg TiO_2NPs/L standards by

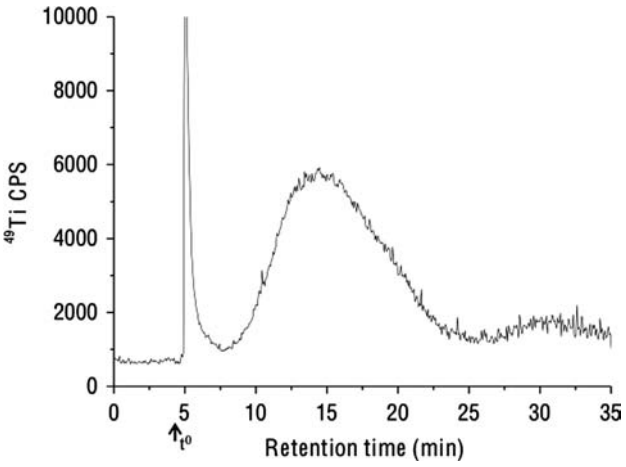


Fig. 4. AsFlFFF–ICP-MS fractograms showing the detection of TiO_2NPs in moisturizing cream. The black arrow (t^0) indicates the void peak.

Table 2
TiO_2 quantification in consumer products.

	Total Ti (mg Ti/kg sample), (w/w (%))[a]	Extracted Ti (mg Ti/kg sample)		TiO_2NPs quantification after AsFlFFF analysis (mg Ti/kg sample), (R (%))[d]	
		Rutile TiO_2NPs standard[b]	Ionic Ti standard[c]	On-line analysis	Off-line analysis
Cosmetic product					
Moisturizing cream (SPF 10)	3865 ± 139, (0.40 ± 0.01)	3770 ± 24	1586 ± 59	3699 ± 145, (98 ± 4)	4336 ± 685, (112 ± 18)[e] 4630 ± 1447, (120 ± 37)[f]
Food					
Sugar glass	938 ± 103, (0.09 ± 0.01)	n.q.	n.q.	n.d.	n.d.
Coffee cream	713 ± 47, (0.07 ± 0.01)	n.q.	n.q.	n.d.	n.d.

n.q.: Ti not quantified.
n.d.: Ti not detected.
The columns indicate mean values ± standard deviation (n = 3).

[a] Ti concentrations achieved after acid digestion of samples and their corresponding Ti percentages.
[b] Ti concentration calculated by FIA–ICP-MS analysis using rutile TiO_2NPs as the calibration standard (Method 1).
[c] Ti concentration calculated by FIA–ICP-MS analysis using an ionic Ti solution as the calibration standard (Method 2).
[d] Recoveries calculated by comparing the Ti concentrations obtained by on-line and off-line analysis with the Ti obtained after aqueous extraction.
[e] Ti concentration found in fractions collected from the AsFlFFF system. FIA–ICP-MS analysis using rutile TiO_2NPs as the calibration standard.
[f] Ti concentration found in fraction collected from the AsFlFFF system. FIA–ICP-MS analysis using an ionic Ti solution as the calibration standard.

ICP-MS prior mineralization of their corresponding fraction collected from AsFlFFF. Our results indicated that the percentages of Ti were close to 12% and 90% in Peak 1 and Peak 2, respectively.

3.7. TiO_2NPs quantification by off-line and on-line AsFlFFF–ICP-MS calibration approach

Development of calibration methods based on the use of standards with similar properties to that of the NPs present in the samples is of great importance for AsFlFFF–ICP-MS analysis. NPs content in the moisturizing cream was calculated using the linear regression equation generated from the calibration method using the rutile TiO_2NPs standard. An average concentration of 3699 ± 145 mg Ti/kg sample was determined (Table 2, column 5). This concentration was similar to the amount of NPs quantified by FIA–ICP-MS analysis and after sample mineralization. Two different calibration approaches, using off-line AsFlFFF–ICP-MS quantifications were evaluated in order to test the success of the external calibration method proposed in this work.

For off-line quantifications, NPs fractions were collected from the AsFlFFF system. Several aliquots were then spiked with increasing amounts of Ti standards (in form of rutile TiO_2 NPs or ionic Ti) into a FIA–ICP-MS system. Variations in TiO_2NPs contents in comparison to the results obtained using on-line calibration were observed (Table 2, column 6). Standard deviations in off-line calibration strategies were higher than in on-line methods, particularly when the ionic Ti standard was added. The discrepancies in the concentrations calculated by off-line and on-line calibration approaches indicate that the on-line external calibration method is more suitable for AsFlFFF–ICP-MS quantification. This way, possible erroneous data due to differences in the efficiency of nebulization and sample handling can be prevented.

4. Conclusions

TiO_2NPs were successfully characterized and quantified using a hyphenated instrumental platform consisting of AsFlFFF with UV detection and combined with ICP-MS. Besides the optimization of the cross flow rate and carrier composition, the ultrasound energy methodology used in NPs stabilization seems to be one of the main parameters controlling the recovery from the system. The optimized experimental conditions provided a quantitative TiO_2NPs recovery from the AsFlFFF system, avoiding drawbacks due to non-specific interactions between NPs and membrane. A method based on focused sonication for preparing NPs dispersion followed by an on-line external calibration strategy, using rutile TiO_2NPs as standards, has been successfully applied. The developed on-line calibration method represents a promising approach for quantitative determination of TiO_2NPs in moisturizing cream without the need of additional strategies or the use of standard calibrates of different chemical nature. Electron microscopy of rutile TiO_2NPs provides very useful information that can be compared with the characterization provided by AsFlFFF–ICP-MS.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.02.029.

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