ABSTRACT: We report the use of inverse supercritical fluid extraction (SFE) and miniaturized asymmetrical flow field-flow fractionation (mAF4) for the preparation and subsequent analysis of titanium dioxide nanoparticles in model and commercial sunscreens. The approach allows for the fast and reliable fractionation and sizing of TiO$_2$ nanoparticles and their quantitation in commercial products. This new method represents a powerful and efficient tool for the verification of nanoparticle content in a wide range of matrixes, as demanded by recently introduced regulatory requirements. Furthermore, the use of carbon dioxide as an environmentally friendly solvent is in line with the increasing need for ecologically compatible analytical techniques.

Recently introduced European Union regulations on cosmetic products require that all ingredients present in the form of nanomaterials be clearly indicated as such in the list of ingredients.$^1$ This requirement calls for the development of comprehensive analytical procedures to ensure manufacturer compliance. The analysis of nanoparticles in complex media, consisting of a complex multicomponent matrix,$^2$ is a multifaceted challenge involving multiple component processes for sample pretreatment, separation of the engineered nanoparticles (ENPs) from the matrix, separation of nanoparticles on the basis of their size, and chemical analysis. To this end, Wagner et al. recently presented such a categorization in a generic sample preparation scheme for inorganic ENPs within complex matrices.$^3$ Such generic procedures, which also integrate appropriate quality criteria to confirm the applicability of the suggested methods, are urgently needed for standardized and systematic development of processes for the separation and analysis of ENPs in complex matrixes. In this respect, the most pressing needs are the development of new analytical techniques for extraction, cleanup, and separation with a view to improving analytical speed, sensitivity, and specificity.$^2$ One of the most challenging components of the Wagner scheme is the reduction in complexity of the ENP containing sample, either through extraction of nanoparticles from their environment or through simplification of the matrix by removal of excipients that interfere with subsequent analysis. There are several techniques that are frequently used for the isolation of nanoparticles from such matrixes, including acid digestion (assisted by heat, sonication, or microwaves),$^3,5,8$ colloidal extraction,$^3,9$ or various treatments with organic solvents.$^7,10,11$ Accordingly, the simplification of sample preparation workflows as well as a reduction in solvent requirements, are highly desirable for the analysis of nanoparticle-containing samples.

To this end, we present a novel method for the analysis of TiO$_2$ nanoparticles in commercial sunscreens, comprising two
analytical procedures. We herein propose the use of inverse supercritical fluid extraction (SFE) to simplify complex matrices containing ENPs while maintaining their particulate nature. To date, the technique of inverse SFE has primarily been used for the isolation of nonpolar pharmaceutical formulations from polar analytes. In a recent publication, we presented proof-of-concept experiments that suggested the utility of the technique as a sample pretreatment tool for nanoparticle-containing samples. Specifically, a single model sunscreen was treated and subsequently analyzed by asymmetrical flow field-flow fractionation (AF4) in addition to UV and MALS (multiance detector) and the use of more sophisticated transmission electron microscopy (TEM) analysis. In the present work, we aim to provide a thorough quantitative analysis of several model and commercial sunscreens loaded with ENPs at varying concentrations. In addition to the measurement of processed materials using mAF4, the ENP content was also analyzed using elementspecific tools such as inductively coupled mass spectrometry (ICP-MS) and inductively coupled plasma atomic emission spectroscopy (ICP-AES). Field-flow fractionation methods are ideal for such measurements due to the gentle forces used to induce analyte retention and prevent particle alteration.

Crucially, the miniaturized AF4 platform reduces both processing times and eluent consumption.

**MATERIALS AND METHODS**

**Samples.** Titanium Dioxide Nanoparticle Standards. A titanium dioxide (TiO2)-nanoparticle dispersion, AERODISP W 740 X (40% w/w, EVONIK Industries, Hanau, Germany) was diluted and prepared according to a protocol described elsewhere to yield a final particle concentration of 0.2 mg/mL.

**Model Sunscreen.** The novel sample preparation method was initially applied to three complex sunscreen models containing different TiO2 nanoparticle concentrations. Creams were produced individually according to protocols described elsewhere. In the final step, 0.5, 2.5, and 12.5% w/w of a AERODISP W 740 X TiO2 nanoparticle dispersion (40% w/w, EVONIK Industries, Essen, Germany), as well as the excipients Dow Corning 1503 (Dow Corning Corporation, Midland, MI, United States) and Euxyl PE 9010 (Schülke & Mayr GmbH, Norderstedt, Germany) were added to each cream, resulting in a TiO2 particle concentration of 0.2, 1.0, and 5.0% w/w, respectively. All creams were homogenized for 5 min at 4000 rpm before being loaded into tubes and stored at room temperature.

**Commercial Sunscreens.** Five different commercial creams were used to assess our sample preparation method: one cream with a sun protection factor (SPF) of 15 (Nivea Cream30), two creams of different brands with a SPF of 30 (Garnier Cream30 and Nivea Cream30), one cream with an SPF of 50 (Coop Cream50), and a sun protection spray with an SPF of 30 (Sherpa Spray30). The first three samples contain TiO2 nanoparticles listed as an ingredient according to European Union legislation, whereas the latter two do not list any nanoparticles as ingredients.

**Sample Treatment.** Supercritical fluids are well-suited for extraction processes due to their minimal surface tension, low viscosities, and gas-like diffusivities, which allow for thorough sample penetration while maintaining the structure of the residual material. The most obvious choice for the current application is supercritical carbon dioxide (scCO2) because almost all chemical excipients found in emulsion-based cosmetic products are highly soluble in CO2. scCO2 is chemically inert as well as being nontoxic and nonflammable and is commonly used in the extraction of small and/or nonpolar molecules from natural materials.

In this regard, common applications include the extraction of essential oils from herbs and spices, the removal of caffeine from coffee beans, and the extraction and analysis of antioxidants, preservatives, and sunscreen agents in cosmetic products. In all these applications, however, the analyte itself is soluble in CO2 with SFE being used to dissolve and remove the analyte from the matrix (upper panel of Figure 1). In this work, however, inverse SFE is used to simplify the matrix by removal of only unwanted components (see lower part of Figure 1). Put simply, the supercritical fluid permeates the matrix, dissolving fatty components and leaving behind a simplified matrix. Once complete, any residual CO2 is simply removed by lowering the pressure below the critical threshold and returning to ambient conditions. The remaining sample material consists of the polar components of the matrix (e.g., thickening agents) along with any nanoparticles present. Residue can be easily rewetted and subsequently dispersed in an aqueous matrix for subsequent analysis.
These procedures have previously been shown to allow SFE followed by the corresponding element-specific evaluation of recovery rates, the detected concentrations were compared to those of the samples prepared by direct detection. For the batch-analysis of samples, two different element-specific detectors were used, namely ICP-AES and ICP-MS. For the evaluation of recovery rates, the detected concentrations were compared to those of the samples prepared by direct microwave-assisted digestion (without any treatment by inverse SFE) followed by the corresponding element-specific method. These procedures have previously been shown to allow recovery rates of 98.2 ± 2.2% for the ICP-AES and 101 ± 2% for the ICP-MS.

Inductively Coupled Plasma—Atomic Emission Spectrometry. Titanium determinations were carried out on a PerkinElmer Optima 3100 XL (PerkinElmer Italia S.p.A., Milano, Italy) ICP-AES equipped with an axial torch, segmented array charge-coupled device detector, and Low-Flow GemCone nebulizer with cyclonic spray chamber for sample introduction and choosing, among the several wave-lengths, the readings at 337.279 nm. The plasma conditions used were an RF power of 1350 W applied to the plasma and flow rates of 15 L min⁻¹ for the plasma gas and 0.5 L min⁻¹ for the auxiliary gas with a nebulizer gas flow of 0.65 L min⁻¹. The sample uptake was 1.5 mL min⁻¹ for each of 3 replicate scans. The diluted standard solutions were prepared from an elemental standard solution (1000 mg L⁻¹ Ti, monoelemental standard solution, Carlo Erba, Italy). Titanium quantification limits were evaluated each time from the calibration curves, and the values ranged between 0.54–0.59 mg L⁻¹.

Inductively Coupled Plasma—Mass Spectrometry. Titanium contents were also determined using an Agilent 7900 (Agilent Technologies, Santa Clara, United States) ICP-MS. Sample introduction was carried out with a concentric quartz nebulizer, quartz glass spray chamber and a quartz glass axial torch. A RF power of 1550 W and an argon gas flow of 15 L min⁻¹ with a 0.9 L min⁻¹ auxiliary gas flow and a carrier gas flow of 1.05 L min⁻¹ were used. The sample uptake flow rate was 1.38 mL min⁻¹ with a stabilization time of 60 s. The titanium isotopes were recorded with an integration time of 0.241 s. All measurements were performed in triplicate in He-mode to remove polyatomic interferences by introducing a helium flow of 43.3 mL min⁻¹ to the collision cell. The calibration was performed with a multielemental standard solution of 1 mg L⁻¹ titanium (CPChem Ltd., Stará Zagora, Bulgaria), and a detection limit of 13 ± 4 µg L⁻¹ was obtained according to DIN32645 (calibration curve method). Prior to analysis, all samples were diluted with a 2% (v/v) HNO₃ solution. The isotopes ⁴⁸Ti and ⁴⁰Ti were always measured simultaneously. However, only the data from ⁴⁰Ti are shown.

Miniaturized AF4 with a Multi-Detector Array, Instrumentation and Carrier Liquid. Samples treated with inverse SFE were further separated and analyzed using an asymmetrical flow-field-flow fractionation system (AF4) from Postnova Analytics GmbH (AF2000 MF, Postnova Analytics GmbH (PN), Landsberg am Lech, Germany), incorporating an autosampler (PN5300), channel thermostat (PN4020), UV (PN3211) and multi-angle light scattering MALS (PN3621, 219 angles) detectors. A miniaturized AF4 cartridge with a tip to tip length of 7 cm (S-AF4-CHA-631, a similar design is further described in ref 18) and incorporating a small 10 kDa regenerated cellulose membrane (20 × 80 mm, Z-263 AF4_MEM-635–10KD) was used for samples of the model sunscreens and a miniaturized 10 kDa polyether sulfone (PES) membrane (20 × 80 mm, Z-AF4_MEM-631–10KD) for the commercial sunscreens. Furthermore, a 350 µm-thick Mylar 267 spacer was used for all measurements. UV detection was performed at 254 nm, and the measured UV signal was used to correlate concentration with particle size. The MALS detector provided the gyration radius of the particles exiting the miniaturized AF4 (mAF4) separation cartridge (calculated using the random coil model). All presented UV data were collected with the UV detector alone, and all radii of gyration data were determined from the angular dependent light scattering signals obtained via MALS detection. The eluent was prepared using filtrated ultrapure water, to which 0.2% (v/v)
RESULTS AND DISCUSSIONS

Method Evaluation with Model Sunscreen. Recovery Rate. One of the most relevant figures of merit when separating ENPs from complex matrices is the recovery rate. This was investigated with ICP-AES and ICP-MS for three model sunscreens that were independently treated with both a standard procedure based on microwave-assisted digestion and inverse SFE.

As shown in Table 1, slightly higher concentrations of TiO2 were obtained for samples processed with scCO2, resulting in recovery rates of 104 ± 4% for the ICP-AES and 115 ± 17% for the ICP-MS. This apparent increase in concentration is most likely due to solvent evaporation during sample preparation for the inverse SFE treatment: the amount of cream to be processed by scCO2 is weighed after deposition on the Teflon cartridge, where it is spread into a homogeneous 200 μm thin layer to allow thorough sample penetration. In the time between sample deposition and cartridge weighing, the sample is prone to evaporation due to the small amount of cream (about 100 mg) and the large exposed surface (600 mm²). This, in combination with the fact that sunscreens are often tailored for fast solvent evaporation (to promote quick absorption by the skin), may lead to mass losses of up to 10%, shifting the nanoparticle w/w concentration toward higher values. This does not occur when performing acid digestion, thereby explaining the small difference in measured concentrations.

Particle Size Evaluation. Another important factor with regard to the applicability of a sample preparation method is its ability to preserve the size distribution of the nanoparticles and prevent the creation of large agglomerates. To assess this, we used a miniaturized AF4 cartridge to perform rapid size separation and subsequent characterization by different detection techniques. With the miniaturized cartridge and the correspondingly low flow rates, solvent consumption could be reduced to less than 25 mL per run, compared to the 100 mL normally used within the standard analytical channel. The complete run time with the miniaturized channel was 45 min, significantly shorter than what is needed when using standard analytical cartridges, especially when taking into consideration flow presetting and flushing. Besides the three model sunscreens, we also measured the pure nanoparticle dispersion used in their fabrication, allowing direct comparison of the size of the original nanoparticles to those remaining in the simplified matrix after treatment with scCO2. These data are shown in Figure 2, where the solid lines depict the UV fractograms for the different model sunscreens and the black dashed line shows the comparative UV data for the original nanoparticle suspension. The red dashed line shows the averaged MALS curve for the pure dispersion, which is in good agreement with the corresponding curves for the model sunscreens. Peak positions in the different fractograms are in close correspondence with only a small shift of the main peak for the treated particles toward larger sizes. These slight shifts are almost certainly due to aggregation. Nevertheless, because larger particles scatter light strongly, even small amounts of aggregates would result in higher signals. Accordingly, the elugrams indicate the presence of only minor aggregates. The very small peak occurring between 15 and 20 min most likely originates from other cream excipients being slightly active in

Table 1. Titanium Dioxide Concentrations Measured after Different Sample Treatments and Subsequent Element-Specific Analysis with Model Sunscreens

<table>
<thead>
<tr>
<th>Sample Preparation: Microwave Digestion</th>
<th>Sample Preparation: Inverse SFE</th>
</tr>
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<tbody>
<tr>
<td>Conc (%)</td>
<td>SD</td>
</tr>
<tr>
<td>Model sunscreen, 0.2% TiO2</td>
<td>0.19</td>
</tr>
<tr>
<td>Model sunscreen, 1.0% TiO2</td>
<td>1.02</td>
</tr>
<tr>
<td>Model sunscreen, 5.0% TiO2</td>
<td>5.17</td>
</tr>
</tbody>
</table>
the selected UV absorption range, as previously noted\textsuperscript{16} and through additional ICP-MS measurements (data not shown). Overall, however, the data clearly indicate excellent preservation of the size distribution of the particles between the pure and treated samples.

**Method Evaluation with Commercial Sunscreens.**

**Recovery Rate.** After the sample preparation efficiency of inverse SFE was thoroughly tested on model sunscreens, the method was applied to commercial sunscreens. For these tests, we purchased a set of five sunscreens and processed them using the same protocol that was used for the three model sunscreen formulations, in addition to microwave-assisted digestion and the treatment with supercritical CO\(_2\) (Table 2). Significantly, the TiO\(_2\) concentrations, determined after microwave digestion with the two methods, are in good agreement, allowing an accurate estimation of the TiO\(_2\) content in the commercial formulations. The values for the CoopCream50 and the SherpaSpray30 (the two creams sold as being TiO\(_2\)-free) gave signals consistently below the detection limit of the respective analytical technique.

An interesting result was observed when using elemental techniques to determine the TiO\(_2\) concentration after inverse SFE. The value obtained with ICP-AES was approximately 79\% of the initial concentration, with little variation among different screens. Conversely, ICP-MS data estimated 52, 37, and 44\% of the initial concentration, with little variation among different screens. The reduced recovery rate for the commercial sunscreens may indicate a severe loss of TiO\(_2\) during sample preparation with inverse SFE. However, it has been shown previously that organic acids can form in the presence of water and scCO\(_2\) and that such acids can diminish the suspension stability of TiO\(_2\) nanoparticles.\textsuperscript{31,32} The treatment with inverse SFE could therefore lead to a modification in the nanoparticle’s surfactant properties, which would result in particle agglomeration. These larger aggregates are unlikely to be completely ionized by the ICP ion source, thereby resulting in lower recoveries at the detector.

Furthermore, the different measurement process parameters of ICP-MS and ICP-AES such as speed and sample uptake could also yield more rapid precipitation for highly aggregated particles in the case of ICP-MS, leading to lower recovery rates compared to those of ICP-AES.\textsuperscript{33}

To further investigate this issue, we performed additional measurements where sunscreens processed using inverse SFE were further treated with microwave-assisted digestion prior to analysis with element-specific tools. This additional mineralization step should allow the proper ionization of all the TiO\(_2\) left after treatment with supercritical CO\(_2\), clarifying whether particles were lost during inverse SFE treatment or not.

The data in Table 3 report the recovery rates for the three nanoparticle-containing commercial samples after employing only inverse SFE and after processing creams with inverse SFE followed by microwave-assisted digestion. As can be seen, the obtained recovery values are higher in the latter case, indicating the issue is not a result of particle loss during inverse SFE but rather due to incomplete ionization of the gently treated samples.

**Particle Size Evaluation.** The aforementioned drop in recovery rate may pose an issue for quantitative measurements. However, one significant advantage of inverse SFE over microwave-assisted digestion is its capability to preserve nanoparticles during pretreatment, thus allowing further investigation. In the case of microwave-assisted digestion, the metals are fully solubilized, thereby destroying their particulate nature and preventing further investigation. On the contrary, the treated particles were lost during inverse SFE treatment or not.

**Table 3. Recovery Rates of the Three Commercial Products Containing TiO\(_2\) Nanoparticles**

<table>
<thead>
<tr>
<th>sample preparation: inverse SFE</th>
<th>sample preparation: iSFE + microwave digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>analytical method: ICP-MS (%)</td>
<td>analytical method: ICP-AES (%)</td>
</tr>
<tr>
<td>NC15</td>
<td>52 ± 16</td>
</tr>
<tr>
<td>NC30</td>
<td>52 ± 16</td>
</tr>
<tr>
<td>GC30</td>
<td>44 ± 7</td>
</tr>
<tr>
<td>Abbreviations: NC15, NiveaCream15; NC30, NiveaCream30; GC30, GarnierCream30.</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Titanium Dioxide Concentrations Measured after Different Sample Treatments and Subsequent Element-Specific Analysis for Five Commercial Sunscreens**

<table>
<thead>
<tr>
<th></th>
<th>sample preparation: microwave digestion</th>
<th>sample preparation: inverse SFE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>analytical method: ICP-MS</td>
<td>analytical method: ICP-AES</td>
</tr>
<tr>
<td></td>
<td>conc (%)</td>
<td>SD</td>
</tr>
<tr>
<td>NiveaCream15</td>
<td>0.99</td>
<td>0.03</td>
</tr>
<tr>
<td>NiveaCream30</td>
<td>1.9</td>
<td>0.2</td>
</tr>
<tr>
<td>GarnierCream30</td>
<td>3.4</td>
<td>0.2</td>
</tr>
<tr>
<td>CoopCream50</td>
<td>&lt;LoD</td>
<td></td>
</tr>
<tr>
<td>SherpaSpray30</td>
<td>&lt;LoD</td>
<td></td>
</tr>
</tbody>
</table>

DOI: 10.1021/acs.analchem.7b04535

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Figures 3A–E show the fractograms of resuspensions from the commercial samples treated with supercritical CO2. In the case of subset A–C, both UV and MALS data show a continuous signal, in line with actual increase in concentration or size and similar to that observed with the model samples. These three creams list, as previously noted, TiO2 nanoparticles as one of their ingredients. The creams not listing TiO2 or any kind of nanoparticles show minimal UV absorption. In those cases, the MALS detector is unable to determine a cohesive sizing curve, as the low scattering signal is insufficient to allow fitting to the model (Figures 3D and E). The small UV signal could be due to excipients other than TiO2 nanoparticles, which may not have been fully removed by inverse SFE and which are also active in the selected UV absorption range.

Figure 3F shows the area under the UV curve for all commercial samples up to a determined diameter of gyration of 100 nm. As the MALS curves for the two later creams, CoopCream50 and SherpaSpray30, never exceed this threshold, no values could be calculated for those samples. Due to the unknown UV absorbance of other cream components and the relatively large deviations between different runs on the same sample (see error bars in Figure 3F), these data cannot be used for an accurate determination of TiO2 content. Nevertheless, the trends are consistent with the results obtained from the element-specific detection techniques, indicating that the method is eminently suitable for a preliminary screening of TiO2 particle content.

Inconsistencies in the recovery rate of ICP-MS compared to ICP-AES, as seen here, are generally not uncommon and have been shown to become more severe if different sample pretreatment are employed. In addition to the variations between the different creams, we observed significant deviations between different aliquots of the same sample. This is also indicated by the standard deviations and percentage ranges shown in Tables 2 and 3 as well as the error bars of Figure 3F, which demonstrate the challenging nature of the analysis of the commercial creams compared to model sunscreens. Results could be improved by extending sample preparation by post-treatment stabilization of the sample, as suggested by Wagner. These additional procedures would stabilize the particles and therefore the analytical outcome as a whole as well as optimize method performance for a specific combination and purpose such as iSFE-mAF4-UV-MALS for the determination of TiO2 particle size distributions or microwave-assisted digestion of the native creams followed by ICP-AES or ICP-MS for the determination of the overall TiO2 content.

### CONCLUSIONS

In this study, we present a novel approach for the analysis of TiO2 nanoparticles in commercial sunscreens that comprises two analytical procedures. In a first step, inverse SFE was used to gently remove sunscreen matrix components, resulting in dried sunscreen strips containing residual TiO2 nanoparticles, which were easily redispersible. With recovery rates ranging between 68 and 110%, ICP-MS and ICP-AES measurements of the commercial sunscreen samples before and after treatment confirmed no significant loss of TiO2 nanoparticles during the sample preparation process.

In a second step, after redispersion in an aqueous solvent, the TiO2 nanoparticles were characterized using mAF4 hyphenated with UV and MALS detection. This approach allowed for fast and reliable fractionation and sizing of the TiO2 nanoparticles and thus an unambiguous determination of the presence or absence of TiO2 particles in commercial sunscreens. The obtained results were in agreement with the data from the element-specific tools and with the label indications for all investigated creams.
The combination of inverse SFE with miniaturized AF4 proved to be a highly powerful and efficient tool for the verification of the nanocontent of commercial sunscreens. Due to its wide applicability, this analytical approach is not solely limited to sunscreens but could also be used with cosmetic formulations containing other inorganic particles such as zinc oxide, ceria, or silver. Furthermore, the method is fully in line with the increasingly compelling demand for environmentally friendly analytical methods due to the use of a mild solvent (scCO₂) in combination with reduced consumption of eluent.

In addition, as a pure sample preparation method, inverse SFE could potentially be applied to other analytical techniques for the investigation of nanoparticles in complex cosmetic matrices, including electron microscopy, nanoparticle tracking analysis or single particle ICP-MS. This renders inverse SFE a universal tool for the preparation of cosmetic samples, significantly contributing to the quest for routine analytical methods for the verification of European Union nanoparticle labeling requirements.

### ABBREVIATIONS

- CO₂: carbon-dioxide
- CO₂: supercritical CO₂
- AF4: asymmetrical flow field-flow fractionation
- mAF4: miniaturized AF4
- SFE: supercritical fluid extraction
- iSFE: inverse SFE
- MALS: multiangle light scattering
- ICP-AES: inductively coupled plasma atomic emission spectrometer
- ICP-MS: inductively coupled plasma mass spectrometer

### REFERENCES