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Detection of nanoplastics in food by asymmetric flow field-flow fractionation coupled to multi-angle light scattering: possibilities, challenges and analytical limitations.

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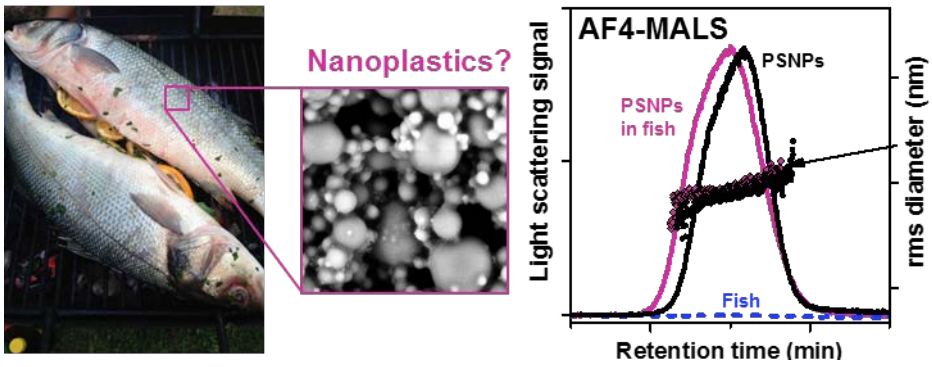
Abstract

We tested the suitability of asymmetric flow field-flow fractionation (AF4) coupled to multi-angle light scattering (MALS) for detection of nanoplastics in fish. A homogenized fish sample was spiked with 100 nm polystyrene nanoparticles (PSNPs) (1.3 mg /g fish). Two sample preparation strategies were tested: acid digestion and enzymatic digestion with Proteinase K. Both procedures were found suitable for degradation of the organic matrix. However, acid digestion resulted in large PSNPs aggregates/agglomerates (> 1 µm). The presence of large particulates was not observed after enzymatic digestion, and consequently it was chosen as a sample preparation method. The results demonstrated that it was possible to use AF4 for separating the PSNPs from the digested fish and to determine their size by MALS. The PSNPs could be easily detected by following their light scattering (LS) signal with a limit of detection of 52 µg /g fish. The AF4-MALS method could also be exploited for another type of nanoplastics in solution, namely polyethylene (PE). However, it was not possible to detect the PE particles in fish, due to the presence of an elevated LS background. Our results demonstrate that an analytical method developed for a certain type of nanoplastics may not be directly applicable to other types of nanoplastics and may require further adjustment. This work describes for the first time the detection of nanoplastics in a food matrix by AF4-MALS. Despite the current limitations this is a promising methodology for detecting nanoplastics in food and in experimental studies (e.g. toxicity tests, uptake studies).

Keywords Nanoplastics, asymmetric flow field-flow fractionation, nanoparticles, multi-angle light scattering, enzymatic digestion.



Introduction

It is well known that oceans and water sources are contaminated with microplastic particles (0.1-5000 µm, microplastics) resultant from the worldwide accumulation of plastic pollution [1–4]. Microplastics can be formed by fragmentation and weathering of larger plastic debris in the oceans, rivers and lakes [1–6]. Microplastics can also be directly transferred to oceans and water sources from waste waters (e.g. fibers from clothes, plastic beads in cosmetics and other products), human activity (e.g. residues from ship industry) or by atmospheric deposition (tyre dust) [1–6]. The three most reported polymer types in microplastics are polyethylene (PE), polypropylene (PP) and polystyrene (PS), which are the most produced plastic polymers (according to production in the U.S.A. in 2012) [2, 3]. It is very likely that nanoplastics (1-100 nm) will also be present in oceans and other water sources, as a result of further fragmentation of microplastics (physical and chemical degradation, due to UV-light exposure for instance), although this has not been demonstrated yet [1–4]. However, the formation of nanoplastics from marine microplastics has already been verified at the laboratory scale under controlled and environmentally representative conditions [7]. Using a homemade solar reactor, Gigault et al. demonstrated *in vitro* that UV-light exposure can result in the progressive degradation of marine PE and PP microplastic fragments, leading to the formation of nanoplastics [7].

Micro- and nanoplastics are considered as an emerging risk in terms of food safety as these particles can eventually end up in the human food chain as a result of their transfer to fish and other sea food (e.g. ingestion of plastic particles by fish) and to marine based products (e.g. table salt, algae) [1–3]. The potential health effects of human exposure to these particles are not yet well understood and can be related to the particles themselves, the chemical substances absorbed to their surface or both [3]. Plastic particles are known to absorb persistent organic pollutants, to contain additives such as plasticizers and have the ability to carry these organic contaminants into marine and freshwater environments [3]. In humans, potential health effects of microplastics are predicted to be confined to the gut and local immune system, as these particles are not likely to cross cell membranes, due to their large size (>1 µm) [3]. Nanoplastics on the other hand, similarly to other nanoparticles (NPs), may be transported across cell membranes and thus could circulate in the blood stream and penetrate in other organs, even the brain [3]. Recently, it was shown in a model study that nanoplastic particles can actually cross the blood brain barrier of fish [8]. In that study, fish (crucian carp) was fed with the freshwater invertebrate *Daphnia Magna* that had previously been exposed to model PS nanoparticles (PSNPs, nominal size 53 nm, positively charged amino-modified PS). The results of this study demonstrated not only the direct uptake of the PSNPs up to the fish through the food chain, but also that the PSNPs entered the brain of the fish, resulting in behavioral disorders, reduced survival rate and thereby affecting the function of natural ecosystems [8].

In nature, microplastics have already been detected in a large variety of zooplanktonic organisms and also in invertebrates (shrimp, bivalves) and vertebrates (fish) [1–3] which are exposed either directly or via lower trophic levels. Microplastics have also been detected in a wide variety of table salt products in different countries [9, 10], and are likely to originate from environmental contamination of the water sources. A few studies have also reported potential contamination of beer [11] and honey [12] with microparticles of plastic origin. These findings have however been recently challenged [13], given that the methodology used could lead to false positives and that the detected particles had not been thoroughly identified as plastics with confirmatory methods (e.g. Raman spectrometry). The methods developed for detection of microplastics in seafood and table salts typically involved three steps: 1 - extraction and/or degradation of the food matrix (e.g. hydrogen peroxide oxidation; acid, base or enzymatic digestion); 2 – detection, quantification and sizing (counting the number of particles by naked eye or using a microscope and classification according to particle size/shape/color); 3 - characterization/identification of the plastics (by Fourier-transform infrared spectroscopy (FTIR), Raman spectrometry or pyrolysis gas chromatography coupled with mass spectrometry (py-GC/MS) [2, 3]).

In contrast to microplastics, there are currently no robust analytical methods for detection and quantification of nanoplastics in seafood and other products. Therefore it is not possible to estimate the oral human exposure via food. In principle, some of the analytical methods used for detecting and characterizing inorganic NPs, like metal and metal oxide NPs, should be applicable for the detection of nanoplastics. Electron microscopy is widely used in polymer science and has been used over the years to characterize several types of nano-sized plastic structures and particles [7, 14]. Also light scattering (LS) based techniques have been exploited to monitor the formation of NPs from fragmentation of plastics, such as dynamic light scattering (DLS) [7] and nanoparticle tracking analysis [15]. These techniques were found to be suitable to detect and characterize nanoplastics in in simple matrices (e.g. salt water). However, detecting nanoplastics in a food matrix is more challenging as it is difficult to distinguish the nanoplastics from the organic food matrix due to the similar density (typically between 0.9 and 1.4 $g.cm^{-3}$[5]) and chemical composition (mainly carbon, hydrogen). In most cases, the procedure would not only require a degradation step of the matrix but also isolation/separation of the nanoplastics from organic residues or other particulates for proper identification of the nanoplastics. In this respect, hyphenated techniques like field flow fractionation and hydrodynamic chromatography coupled to multiple detectors (e.g. multi-angle light scattering, MALS or UV-vis absorbance) are quite attractive as they allow to separate small molecules (e.g. matrix residues), particles and macromolecules in a considerable size range (around 1 to 1000 nm) ahead of the detection by LS or absorbance [16]. Furthermore, these techniques are widely used to analyze polymers and polymeric particles [16]. Recently, asymmetric flow field flow fractionation (AF4) has been successfully exploited to separate and analyze a polydisperse PS particle sample containing nanoplastics (size range 80 to 600 nm) in aqueous solution [17].

In this paper we study the feasibility of AF4 coupled to MALS for the detection of nanoplastics in a complex food matrix (fish fillet) after suitable sample preparation. Two types of sample preparation, acid and enzymatic digestion, were compared. The selected nanoplastics were PSNPs with a nominal size of 100 nm and PE particles with a broad size distribution including particles in the nano-size range (1 to 100 nm). Herein, we show the different methodological steps necessary to analyze nanoplastics in fish by AF4-MALS. We also discuss the challenges that had to be addressed during method development, the limitations of such methodology, as well as future considerations to be taken when developing detection methods for nanoplastics in fish and other food items.

Materials and methods

Materials

Ultrapure water (18.2mΩ/cm) was obtained from a Millipore Element apparatus (Millipore, Milford, MA, USA) and used throughout the work. ReagentPlus sodium dodecyl sulfate (SDS) with ≥ 98.5% purity, sodium bicarbonate ($NaHCO_3$) with 99.5 to 100.5 % purity and sodium azide with ≥ 98 % purity were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitric acid (67–69 %) of PlasmaPURE quality was obtained from Science (Quebec, Canada). Fisherbrand™ FL-70™ Concentrate (FL-70) was acquired from Fisher Scientific (Pittsburgh, MA, USA). Ethanol with (96 % purity) and Suprapur® hydrogen peroxide (30%) was purchased from Merck (Darmstad, Germany). The fish sample was taken from European seabass (*Dicentrarchus labrax)*, obtained from Ecloserie Marine de Gravelines Ichtu, Graveline , France. The fish were kept for seven weeks in seawater tanks at the National Institute of Aquatic Resources at the Technical University of Denmark on a diet from BiomarA/S as part of a trial of the European Union ECsafeSEAFOOD project (http://www.ecsafeseafood.eu/). The sample contained single fillets from three fish that were homogenized by a blender. The fish homogenate was received frozen and was stored in a freezer throughout the experiments.

Nanosphere™ 3000 Series PSNPs (nominal diameter 100 nm, 100 ± 3 nm NIST traceable diameters as determined by transmission electron microscopy by the supplier) and Dye Red Aqueous fluorescent spherical nanoparticles (FlPSNPs) (nominal diameter 100 nm, peak excitation and emission wavelength of 542 nm and 612 nm, respectively) were obtained from Thermo Fischer Scientific (Fremont, CA, USA). Both PSNPs and FlPSNPs were provided as stable aqueous dispersions with an approximate concentration of 1 % solids (10 mg/mL). In the case of FlPSNPs the use of a proprietary surfactant for dispersion was mentioned in the product's description. Polyethylene Nanospheres (PE particles) with a density of 0.95g/cm³ and particle sizes between 200 and 9900 nm were obtained from Cospheric LLC (Santa Barbara, CA, USA) as dry powder.

For enzymatic digestion the commercial protease Proteinase K from *Engyodontium album* was used (Sigma-Aldrich St. Louis, MO, USA). Proteinase K is a serine protease with a molecular mass 28 930 Da, a broad pH range of 7.5 to 12 and a temperature optimum of 37°C regarding the enzyme activity. As accumulation wall in the AF4 cell PES membranes (Nadir® UP010P) with a molecular weight cut-off of 10 kDa were purchased from Microdyn-Nadir (Wiesbaden, Germany, Lot no. 230864).

Methods

Preparation of PE lower size fraction by centrifugation

A 10 mg/mL stock suspension of the PE particles was first prepared by dispersing 50 mg of PE powder into 5 mL ethanol. The powder was dispersed by mixing with a vortex mixer until an opaque white suspension was achieved. To obtain a lower-size fraction the PE stock suspension, a centrifugation procedure was developed to remove the larger size particles (> 1µm). A 3-18K centrifuge from Sigma Laborzentrifugen (Osterode, Germany) with an 11180 swing bucket rotor was used. Sample tubes were 15 ml Sarstedt (Nümbrecht, Germany) tubes. For calculating the time t_s that it takes for a spherical PE particle of diameter d = 1 µm to travel in ethanol from the surface of the liquid in a tube (fill-up point r_{in} = 11.6 cm; 5 mL mark) to a selected distance below the surface (sampling point r_{out} = 13.6 cm; 2 mL mark) at a constant force (revolutions per minute, rpm = 4700) the following equation previously described by Bernhardt [18] was used:

$$t_s = \ln\left(\frac{r_{out}}{r_{in}}\right)\frac{18\eta}{4\pi^2\Delta\rho d^2\left(rpm/60\right)^2}$$

The density difference of the particles compared the medium was $\Delta\rho$ = 0.16 and the viscosity of the medium (ethanol) was η = 0.001095 Pa·s. A size cut-off of 1 µm was chosen in order to have an adequate size range for AF4 analysis (1 to 1000 nm). Based on these calculations, the PE stock suspension was centrifuged for 1 min at 4700 rpm. After centrifugation, a white pellet was clearly visible in the bottom of the tube. Three milliliters of the top liquid layer were then collected and homogenized by vortex-mixing. According to the calculations, this supernatant consisted of PE particles with a size below 1 µm. The assumption was confirmed by scanning electron microscopy (SEM). Only this lower size fraction was used for the AF4 experiments.

Acid digestion

For acid digestion, 30 mg of the fish homogenate were directly weighted into disposable standard glass vials (Wheaton® 15x46 mm, cap 13-425). The PSNPs were incorporated into the fish samples by dispensing the stock suspension of PSNPs aqueous dispersion onto the fish homogenate just before homogenization by vortex mixing and acid digestion. A volume of 25 µL of 10 mg/mL PSNPs was spiked to the fish homogenate corresponding to a spiked mass of 8.3 mg PSNPs / g fish. In non-spiked fish samples 20 µL of ultrapure water was added instead. A control PSNPs sample (no fish) was also prepared, where the same amount of PSNPs (25 µL of 10 mg/mL PSNPs) was added to 30 µL ultrapure water. Subsequently, the samples were digested in a mixture of 0.250 ml concentrated nitric acid and 0.125 ml hydrogen peroxide in a microwave oven (Multiwave 3000, Anton Paar GmbH, Graz, Austria) equipped with a 64MG5 rotor at elevated temperature and pressure (~ 140°C; max. 20 bar). After digestion the samples were diluted six times with 0.05 % (w/w) SDS to a final sample weight of 2.5 g.

Enzymatic digestion

Prior to enzymatic digestion, the frozen fish homogenate was mixed with the same mass of ultrapure water (1:1) using an Ultra-turrax® D25 homogenizer (IKA Works, Inc., Wilmington, NC, USA) to obtain a viscous slurry for further sub-sampling. The samples were then taken from the fish slurry and weighted directly into vials. The PSNPs (FlPSNPs or non-fluorescent PSNPs) were incorporated into the fish by dispensing the required amount of PSNPs aqueous dispersion onto the fish slurry just before homogenizing by vortex mixing and enzymatic digestion. This spiking procedure is described in more detail below. If necessary, the PSNPs dispersions were diluted with ultrapure water prior to spiking to the fish. Before use, the 10 mg/mL PSNPs stock dispersions were gently mixed by inverting the bottles and dispersed by low power bath sonication (10-15 seconds), as recommended by the manufacturer. A previously developed enzymatic digestion procedure was modified and applied [19]. Briefly, the enzyme solution was prepared by dissolving Proteinase K to a final concentration of 3 mg/mL in 47 mM $NaHCO_3$ (pH 7.7) containing 5 mg/mL SDS and 0.2 mg/mL sodium azide (antimicrobial agent). Concentrated nitric acid was used for adjusting the pH of the $NaHCO_3$ solution. A portion of 0.1 g fish slurry was weighed. In spiked samples, 65 µL of 1 mg/mL PSNPs (corresponding to a spiked mass of 1.3 mg PSNPs / g fish) and 85 µL of ultrapure water were then added to the reaction tubes. In non-spiked fish samples 150 µL of ultrapure water was added instead. Subsequently, 1 mL of the Proteinase K solution was added and the samples mixed thoroughly prior to enzymatic digestion. The resulting ratio between enzyme and initial mass of fish (50% of the fish slurry) was approximately 1:17 m/m. The mixture was incubated overnight (16 to 17 h) at 37°C in a water bath using continuous stirring.

For DLS analysis, the spiked particle mass was increased about 17-times to 23 mg PSNPs / g fish to assure detectability of the PSNPs. The increase of PSNP concentration was necessary since there was no separation procedure ahead of the analysis like in the AF4 experiments. Thus, any remaining organic fragments left after digestion contributed to the light scattering signal and superimposed the signal of the PSNPs.

For preparing the fish spiked with PE particles for AF4-MALS analysis, the fish slurry contained a 1:10 fish to water mass ratio. In comparison to the PSNP experiments, the mass of fish was decreased by a factor of ten

to assure the injection of the same mass of fish (200 µg) in the 50 µL injection volume, but without diluting the PE particles (in case of PSNPs, samples were diluted ten times prior to injection to AF4). The aim was to achieve the highest possible PE particle concentration in fish. It was not possible to use higher spiking masses of PE particles, as no more concentrated stock suspension was available. The quantities of the components for the enzymatic digestion were as follows: 0.1 g fish slurry (1:10), 300 µL of PE particles (lower size fraction), and 1 mL of Proteinase K solution. In non-spiked fish samples ethanol was added instead of the PE particles. The mixture was incubated at 37°C in a water bath for 30 min.

Optical microscopy

After enzymatic digestion, fish samples were analyzed by optical microscopy to evaluate if the enzymatic degradation was successful. For comparison purposes a sample containing fish slurry diluted in enzyme buffer (same components as used for enzymatic digestion but with no enzyme) was also prepared. The samples were placed on glass slides and examined using a Leica DMR optical microscope (Leica Microsystems, Wetzlar, Germany) equipped with a DFC295 digital camera (Leica Microsystems, Wetzlar, Germany). Optical micrographs were acquired using the Image Analysis software Image-Pro Plus version 7.0.1.658 (Media Cybernetic, Inc., Rockville, MD, USA).

DLS measurements

The PSNPs and FlPSNPs dispersions, and fish digests were characterized by DLS for information on the hydrodynamic diameter (Zetasizer, Malvern Instruments, UK). Prior to the measurement, the instrument was warmed up for at least 30 minutes. The measurements were carried our using a dust-free 12.5 × 12.5 × 45-mm UV-transparent disposable cuvette (Sarstedt). The measurement volume was 50 µL. An angle of detection of 173° was chosen and the measurement was performed at a temperature of 25°C, which was achieved following equilibration for 1 minute. Three consecutive measurements were performed using a minimum of 11 runs of 10 s each. The laser power (attenuator index) was determined automatically by the instrument. The DLS characteristics of the measured sample, given as Z-average (Z_{ave}) (intensity-weighted harmonic mean diameter) and polydispersity index (PDI), were determined by averaging the result of the repeated measurements (N=3). The PSNPs and FlPSNPs dispersions were diluted in ultrapure water in order to achieve the required concentration. No dilution was done in the case of the fish digests.

SEM measurements

For analyzing the PE dispersions, 10 µL of each sample was transferred to a SEM substrate placed on a metal stub and allowed to dry at room temperature prior to SEM imaging. The examinations were carried out using a Phenom Pro SEM system (Phenom World, Eindhoven, the Netherlands), operated at an acceleration voltage of 10 kV.

AF4-MALS/fluorescence analysis

The AF4 system used in this study consisted of an Agilent 1200 series autosampler (G1329A), a high performance liquid chromatography pump (G1311A) (Agilent Technologies, Santa Clara, CA, USA), an Eclipse™ 3 AF4 flow control module, and a short channel-type AF^4 separation channel (Wyatt Technology Europe GmbH, Dernbach, Germany).The channel had a trapezoid shape with a length from inlet to outlet of 172 mm, a length from inject port to outlet of 152 mm and a width of 24.0 mm at the inlet and 3 mm at the outlet port. The width at the sample inject port was 21.5 mm. The area of the accumulation wall was 2363 mm^2. The AF4 separation program was adapted from [19, 20] and is listed in **Table 1**. AF4 carrier liquids were produced by dissolving $NaHCO_3$ in ultrapure water or by dilution of FL-70 concentrate in ultrapure water. Concentrated nitric acid was used for adjusting the pH of the $NaHCO_3$ carrier liquid. The pH value of the $NaHCO_3$ carrier liquid was controlled during the experiments and was found to be in the interval 7.7 to 7.9 throughout the experiments.

Prior to injection into the AF4 system the enzymatically digested fish samples spiked with PSNPs or FlPSNPs were diluted ten times in ultrapure water. The final concentration of PNSPs or FlPSNPs in the injected

samples was 5.2 µg/mL. Typically, a volume of 50 µL was injected into the AF4 channel corresponding to an injected mass of 260 ng PNSPs or FlPSNPs and an injected fish mass of 200 µg. Pristine PSNPs and FlPSNPs in ultrapure water were analyzed after dilution to a concentration of 5.0 µg/mL, corresponding to an injected mass of 250 ng PNSPs or FlPSNPs (50 µL injection volume).

The lower size fraction of PE particles (pristine particles in ethanol) was analyzed as such. The mass concentration of PE particles in this fraction was unknown. The injection volume of PE particles was adjusted to 10 µL, as this resulted in an AF4-LS peak with a similar height as the one obtained for the PSNPs (260 ng injected mass). The fish samples spiked with PE particles were not diluted prior to injection either. For these samples, a 50 µL injection volume was used in order to have a similar injected mass of particles as in the case of pristine PE particles (particles are diluted approximately five times during enzymatic digestion). The injected fish mass was 200 µg.

Following separation by AF4 various detectors were used to collect information about the eluting fractions. A DAWN® HELEOS™ (Wyatt Technology Europe GmbH, Dernbach, Germany) MALS detector with 17 observation angles operated with a linear polarized laser light at 658 nm was used to record the LS signal. The MALS detector was set to a sampling time interval of 1 s per data point. Data from the LS detectors was processed using the ASTRA V software (version 5.3.2.15, Wyatt Technology Corporation, Santa Barbara, CA, USA). The root mean square diameter (rms) was determined using a 1st order Zimm model for the (Fl)PSNPs and 3rd order Debye model for the PE particles, which were found suitable for fitting of the experimental data. The Zimm model is appropriate for size fitting of spherical particles like the (Fl)PSNPs [21]. The Debye model was used for fitting in the case of the PE particles because of its robustness and fitting capabilities for both spherical and non-spherical particles [21]. An Agilent series 1100 fluorescence detector (Agilent G1321A, FLD) was used to record the fluorescence emission signal from the FlPSNPs (excitation wavelength = 542 nm, emission wavelength 612 nm, selected according to the specifications of the FlPSNPs supplier).

Table 1 AF4 separation program for separation of PSNPs, FlPSNPs or PE particles (adapted from [19, 20]).

Step	Duration (min)	Mode	Cross flow rate (mL/min)
1	2	Elution	0.5
2	1	Focus	-
3	2	Focus + injection	-
4	5	Focus	-
5	30	Elution	0.5
6	5	Elution	-
7	5	Elution + injection	-

Carrier liquid:

- 0.47 mM $NaHCO_3$, pH 7.7 to 7.9 for experiments with PSNPs and FlPNSPs
- 0.025 % (v/v) FL-70 for experiments with PE particles

Membrane material: PES
Membrane cut-off: 10 kDa
Spacer height: 350 µm
Detector flow rate: 1.0 ml/min
Injection flow rate: 0.2 ml/min
Focus flow rate: 0.5 ml/min

Results and discussion

Selection of sample preparation procedure

Lean homogenized fish fillet was selected as a starting model system to spike nanoplastics considering that nanoplastics may migrate to different organs and tissues of the fish after ingestion. For initial development of the sample preparation and the AF4-MALS method, well characterized monodisperse PSNPs of 100 nm nominal size were used and spiked to the fish. According to the supplier, the PSNPs were stabilized with trace amounts of a surfactant to inhibit agglomeration and promote stability. The type of surfactant was not disclosed (proprietary information).

Two sample preparation strategies were then tested for degradation of the fish matrix prior to analysis by AF4-MALS: acid digestion and enzymatic digestion with the protease Proteinase K. Acid digestion was tested as it is has been used as sample preparation procedure for the analysis of microplastics in bivalves [2, 22, 23]. As expected the acid treatment was successful for complete digestion of the fish resulting in a clear solution with a faint yellow color (**Fig. S1a,** see Electronic supplementary material (ESM)). However, the acid treatment led to agglomeration of the PSNPs, as indicated by the presence of visible white particulates in the spiked fish samples (**Fig. S1b**, see ESM). Similar particulates could also be seen after acid digestion of six times diluted PSNPs suspension (**Fig. S1c**, see ESM), suggesting that the PSNPs were destabilized in the acidic environment. One cannot exclude either that the acid digestion resulted in partial chemical oxidation of the PSNPs. In fact, microwave-assisted acid digestion with nitric acid has been exploited in the past to degrade PS and other polymers for chemical analysis of embedded trace elements [24].

The other tested sample preparation strategy was enzymatic digestion with Proteinase K, adapting a protocol which had been previously applied with success for the analysis of silver NPs (AgNPs) in chicken meat by AF4 coupled to inductively coupled plasma-mass spectrometry (ICP-MS) [19]. Such enzymatic digestion procedure allowed moderate experimental conditions in terms of pH and temperature, which were suitable for subsequent AF4 analysis. Also, it was a milder procedure compared to e.g. acid, basic digestions or chemical oxidation which can degrade some of most sensitive plastics [2, 23].

After 30 min of digestion of the fish with Proteinase K, an opaque white homogeneous solution was obtained, indicating that most of the fish matrix had been degraded (**Fig. S2**, see ESM). To verify the effectiveness of the digestion procedure and to ensure the fish matrix was completely digested, optical microscopy was performed on a non-digested and on the digested fish samples. The obtained microscopy images of the non-digested sample showed the presence of large fragments and fibers with sizes in the range 50 to 1000 µm (**Fig. S3a**, see ESM). Such fragments were no longer visible in the digested fish sample (**Fig. S3b**, see ESM). This was observed for both non-spiked fish samples and for fish samples spiked with PSNPs.

DLS was used to study any possible effects of enzymatic digestion on the agglomeration/aggregation state of the spiked PSNPs and to check for the presence of smaller organic matrix residues (which would not be visible by optical microscopy).

The pristine PSNPs showed a monodisperse particle size distribution (**Fig. S4**, see ESM) with a Z_{ave} mean size of around 116 nm (PDI = 0.023). The obtained particle size distribution was similar after enzymatic treatment of the pristine PSNPs in the absence of fish (Z_{ave} of 107 nm and PDI = 0.009, **Fig. S4**, see ESM). This demonstrated that the enzymatic digestion procedure did not alter the size or the agglomeration state of the PSNPs. In the presence of fish, the particle size distribution was slightly broader (PDI = 0.131) and shifted to larger particle sizes (Z_{ave} of 126 nm) in comparison to the pristine PSNPs. These changes were probably due to the presence of smaller organic fragments present from the digested fish matrix (e.g. protein residues or other biomolecules) that also contributed to the LS signal. This was verified by analyzing a sample of digested fish that did not contain PSNPs. A broad peak was obtained with a mean of 231 nm

(PDI = 0.244). In none of the investigated samples, particles larger than 1 µm were detected. Consequently, enzymatic digestion was selected a suitable sample preparation method for the following AF4-MALS analysis.

Detection of PSNPs in fish by AF4-MALS

For initial method development it was decided to use fluorescent PSNPs (FlPSNPs) to have a selective signal (fluorescence) for identification of the nanoplastics. FlPSNPs with the same nominal size (100 nm) and from the same manufacturer (Thermo Scientific) as the non-fluorescent PSNPs were selected. DLS measurements confirmed that both NPs had the same hydrodynamic particle size distributions (Z_{ave} values of 116 and 113 nm for non-fluorescent PSNPs and FlPSNPs, respectively, **Fig. S5**, see ESM).

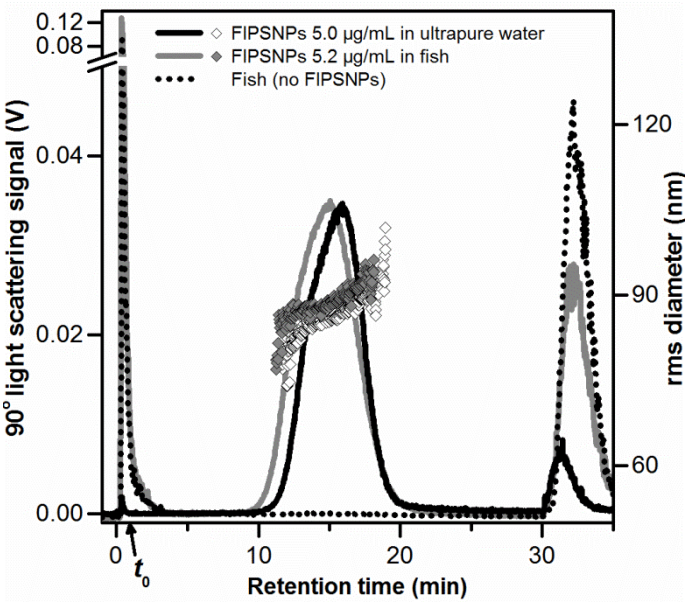


Fig.1 AF4-LS fractograms obtained for the pristine FlPSNPs in ultrapure water, fish spiked with FlPSNPs and non-spiked fish using 47 mM $NaHCO_3$ as a carrier liquid. The rms diameters determined for the pristine FlPSNPs in ultrapure water and for the spiked fish digest are shown as white circles and gray diamonds, respectively. The AF4-MALS measurements were performed using the same AF4 membrane.

The initial AF4-MALS method was developed based on previous experience [19, 20] and first tested for analysis of the pristine FlPSNPs in ultrapure water. The injected mass of FlPSNPs of 250 ng (resulting from the injection of 50 µL of 5.0 µg/mL fPSNPs diluted with ultrapure water) was selected based on previous experience for achieving an adequate AF4-LS signal. The AF4-LS fractogram obtained for the pristine FlPSNPs in ultrapure water is shown in **Fig. 1** (black continuous line). A narrow single elution peak (full width at half maximum / FWHM of 4.5 min) was observed at retention time t_r = 15.9 min, corresponding to the elution of FlPSNPs (main elution peak). After t_r = 30 min the cross-flow rate was set to 0 mL/min and a smaller peak (corresponding to 10 % of the main elution peak area) was observed at t_r = 31.3 min, corresponding to the elution of retained FlPSNPs particles (release peak). The determined rms diameters at the main elution peak were in the interval of 77 to 92 nm (white circles in **Fig. 1**), which was in agreement with the theoretical ratio of rms and geometric diameter for a solid sphere of 0.77. The rms diameters increased linearly with retention time, confirming a proper elution of the FlPSNPs in the AF4 channel (i.e. according to AF4 theory). As expected, a narrow elution peak was also obtained with the fluorescence emission detector (black line in **Fig. S6**, see ESM). The retention time (t_r = 15.9 min) and width (FWHM of 4.8 min) of the elution peak were in agreement with the LS based signal, confirming the fluorescence signal could also be used to monitor the elution of the FlPSNPs. The suitability of AF4-MALS for the detection of

PSNPs is not surprising as this type of NP size standards is typically used for size calibration of AF4 channels [20, 21, 25].

Fish spiked with FlPSNPs (spiked sample concentration 1.3 mg PSNPs / g fish) and enzymatically digested was then analyzed by AF4-MALS using the same separation method. The injected mass of FlPSNPs was 260 ng (injection volume 50 µL). The AF4-LS fractograms obtained for spiked and non-spiked fish are also shown in **Fig. 1** (gray continuous and black dotted line, respectively). The main elution peak obtained for the spiked fish sample was similar to the one obtained for the pristine FlPSNPs in terms of peak area, width and shape (see **Fig. 1** and **Table 2**). There was only a small difference in retention time between the two samples as the FlPSNPs eluted slightly earlier in the digested fish sample than in ultrapure water (t_r = 15.0 min and 15.9 min, respectively). Similar retention times were obtained for the main FlPSNPs elution peaks in AF4-fluorescence fractograms (t_r = 14.6 min and 15.9 min for FlPSNPs in ultrapure water and in fish, respectively). A change in AF4 elution behavior in presence of enzymatic digests was also observed by Loeschner et al. for AgNPs in chicken meat [19]. Also there, AgNPs were shown to have an earlier AF4 elution when enzymatically digested meat was present. The authors demonstrated that this was not caused by changes in the AgNP particle size but most likely due to either changes of NP surface and/or membrane surface properties or disturbance of the relaxation of the AgNPs during focusing in the presence of peptides from enzymatically digested meat [19]. In fact, the rms size data retrieved in our study did not indicate any change in FlPSNPs particle size in the presence of fish. The determined rms diameters (80 to 90 nm) obtained FlPSNPs in fish were very similar to the ones obtained for the pristine FlPSNPs in water (**Fig. 1**, rms diameters shown as grey diamonds and white circles). This confirmed that the enzymatic sample preparation did not affect the particles in terms of size or agglomeration state.

The AF4-LS fractogram obtained for the spiked fish sample showed a clear void peak in the early phase of elution (t_r = 0 to 4 min), which was not the case for the pristine FlPSNPs (**Fig. 1**). A void peak of similar magnitude was also visible in the non-spiked fish sample, indicating that the void peak signal was probably caused by enzymatic digestion residues that were not retained during separation and hence eluted at the void time t_0. There were also differences in the release peaks obtained for spiked and non-spiked fish samples, which were considerably higher in magnitude compared to the release peak for pristine FlPSNPs (the release peak area was more than six times higher for non-spiked fish samples and more than nine times higher for spiked fish samples). This was probably also caused by enzymatic digestion residues that contributed to the LS signal and were retained during the AF4 separation. In fact, our initial DLS results (for non-spiked fish) had already indicated that after enzymatic digestion, light scattering organic residues (50 to 1000 nm in size) were still present in the sample. Additionally, in the AF4-fluorescence fractogram obtained for the spiked fish (red line in **Fig. S6**, see ESM), there was no fluorescence signal at the release peak area. This also confirmed that it was not the FlPSNPs but digestion residues that contributed to the elevated LS signal at the release peak. In contrast to the void and release peak areas, no elevated LS signal was observed in the elution time range of the main peak for the non-spiked fish sample. This confirmed that the AF4-LS elution peak obtained for the spiked fish sample was resultant only of the LS signal coming from FlPSNPs and that the FlPSNPs could be clearly separated from other organic digestion residues.

As next step, the limit of detection (LOD) and linearity for FlPSNPs mass concentration were determined. In a simplified approach, a fish sample containing 1.3 mg FlPSNPs/g fish was injected with increasing injection volume (2, 5, 25 and 50 µL). The corresponding injected masses of FlPNSPs ranged from 10 to 260 ng and the injected masses of fish from 8 to 200 µg. The mass ratio between FlPSNPs and fish was constant (1:770) (see **Table 2**). This approach was used because it was found that the fish matrix had no contribution to the LS signal in the elution time range of the main peak, i.e. that the signal from the FlPSNPs was independent of the mass of injected fish. Down to an injected mass of 10 ng FlPNSPs, it was possible to distinguish the elution peak of the FlPNSPs from the background. This would correspond to an LOD for 100 nm FlPSNPs of 52 µg particles/g fish or $9.4 \cdot 10^{10}$ particles/g of fish if the injected mass of fish was 200 µg (highest mass tested in this study). To further decrease the LOD, the injected mass of fish would have to be increased

above 200 µg. Based on previous experience the injected mass of enzymatically digested biological tissue can be increased up to 1 – 2 mg until channel overloading effects are observed, which means that the LOD could theoretically be decreased by a factor of five to ten. The calculated LOD is specific for 100 nm PSNPs as used in this study. The LOD is expected to be higher for smaller PSNPs as the intensity of scattered light decreases with decreasing diameter of the particles to the power of three (for a fixed particle mass concentration). Already knowing the challenges related to the detection of nanoplastics in a complex biological matrix we decided to focus on the upper end of the nanoscale for initial method development. Future work should be done to evaluate the LOD for smaller PSNPs.

Table 2 Summary of the AF4-LS results obtained for FlPSNPs and non-fluorescent PSNPs in ultrapure water and when spiked to fish including AF4-LS peak area, full width at half maximum values (FWHM) and retention time t_r (main elution peak mode). The AF4-LS measurements were performed using the same AF4 membrane.

Sample	Injected mass of FlPSNPs (ng)	Injected mass of fish (µg)	Injected volume (µL)	Peak area (V·min)	FWHM (min)	Retention time t_r (min)
FlPSNPs 5.0 µg/mL in ultrapure water	250	N/A	50	0.162	4.5	15.9
FlPSNPs 5.2 µg/mL in fish,	260	200	50	0.183	5.1	15.0
	130	100	25	0.088	5.0	15.8
	26	20	5	0.016	4.9	16.5
	10	8	2	0.005	4.7	17.5
Non-fluorescent PSNPs 5.0 µg/mL in ultrapure water	250	N/A	50	0.136	4.0	16.2
Non-fluorescent PSNPs 5.2 µg/mL in fish	260	200	50	0.138	4.8	15.5

As mentioned earlier there are no reports of occurrence of nanoplastics in real samples, so it is not straightforward to evaluate how relevant this LOD would be in the context of screening of nanoplastics in real food samples. There is some occurrence data of microplastics in seafood, but typically the results are reported as the number of microplastics per item or per gram of wet tissue . A recent report from the European Food Safety Authority (EFSA) summarized the findings of the previous occurrence studies of microplastics in seafood, namely in bivalves, where the occurrence of microplastics was highest [2]. The average number of microplastic particles found in bivalves was 0.2 to 4 /g wet tissue [2, 20, 26–29]. If one assumes spherical PS particles and a particle density of 1.05 $g.cm^{-3}$, this corresponds to 0.01 to 0.3 ng plastics/(g wet tissue) for 5 µm particles (lower-size range of microplastics found in bivalves [2]) and 13.7 to 275.9 mg plastics/(g wet tissue) for 5000 µm particles (upper size range found in bivalves [2]). Our AF4-LS method allows detecting 52 µg of PS per gram of wet tissue, which is in between the values estimated for 5 µm and 5000 µm microplastics. The strong dependence of particle mass on particle size (mass is proportional to the size to the power of three) and the low number of studies with size classification information makes it difficult to evaluate the relevance of this LOD value.

The AF4-LS peak area was found to depend linearly on the injected mass of FlPSNPs in the tested range of 10 to 250 ng (**Fig. S7**, see ESM). The shape of the different AF4-LS fractograms obtained for the different injected masses was similar (**Fig. S8**, see ESM). The retention time decreased with increasing mass of injected FlPSNPs (from t_r = 17.5 min for 10 ng to t_r = 15.0 min for 260 ng), which was most likely related to the increasing mass of injected organic material / fish digest. This organic material could lead to changes of

NP surface and/or membrane surface properties (resulting in increased particle/membrane repulsion and consequently earlier elution) as well as disturbance of the relaxation of the FlPSNPs during focusing. Interestingly, the retention time of the pristine FlPSNPs (t_r = 15.9 min) was close to the retention obtained for FlPSNPs in fish at 130 ng injected mass (t_r = 15.8 min). Further experiments with replicate measurements are required to investigate this effect. The retrieved rms diameters (80 to 90 nm) confirmed that the observed shifts in retention time were not due to changes in particle size in the different experiments (data not shown). More importantly, we could retrieve rms diameters down to the lowest injected mass of FlPSNPs (10 ng), meaning that independent of the observed shifts in retention time correct size determination by MALS was possible.

Finally, the method was applied on non-fluorescent PSNPs. The obtained results for both FlPSNPs and non-fluorescent PSNPs (**Fig. S9,** see ESM and **Table 2**), were comparable in terms of AF4-LS peak shape, peak area, retention time and obtained rms diameters, showing that the method was equally applicable for these two types of PNSPs. The AF4-LS channel recovery was determined relatively to AF4 injections of known amounts PSNPs and where no cross-flow field was applied. This recovery value provides an indication on the possible loss of particles occurring during the AF4 separation procedure (e.g. to the membrane). The AF4-LS channel recoveries determined for Fl-PNNPs and non-fluorescent PNSPs in the spiked digested fish samples were in the 60-70 % range. We expect that higher recoveries can be achieved (values close to 100 %) upon optimization of the AF4 separation conditions (e.g. buffer concentration, ionic strength, etc.).

Detection of polyethylene nanoparticles

Nanoplastic particles that result from fragmentation/wearing of plastics can be expected to have different properties than the so far studied monodisperse spherical PSNPs. In the already mentioned study by Gigault et al. on the controlled UV-degradation of marine PE and PP microplastic, the detected nanoplastics were characterized by transmission electron microscopy. The particles were found to have a very polydisperse size distribution (2 to 500 nm) and to be present in different shapes [7]. Besides being different in terms of size distribution (broad) and particle shape (irregular), nanoplastics found in nature might also have other surface properties (no surfactant is present) and chemical composition (PE, PP, etc.) than the studied PSNPs.

To evaluate the suitability of the developed method to analyze also other types of nanoplastics, PE particles were studied. Among the different plastic types, PE was selected as it is the most common plastic, comprising more than 40 % of the produced plastics (according to the industrial production in the US, in 2012) [3]. To the best of our knowledge, there are currently no PENPs (i.e. PE particles with a size in the range of 1 to 100 nm) commercially available. The only PE product with nominal particle sizes close to the nanoscale was the one that was obtained from Cospheric (www.cospheric.com) for this study. The acquired PE particles were hydrophobic and had a broad size range ranging from 200 to 9900 nm according to the manufacturer (based on laser diffraction measurements). SEM analysis of the original PE sample (**Fig. 2a**) confirmed the presence of spherical particles in a very broad size range (going from a few hundred of nm to several microns). The broad size range was not suitable for AF-LS analysis and a separation of the particles < 1µm was required. A centrifugation method was developed to isolate the smaller size fraction of polyethylene particles (see Materials and Methods). Ethanol was used as the solvent for centrifugation, as a solvent with a lower density than the density of the PE particles (0.95 g cm^{-3}) was required. In the lower size fraction recovered after centrifugation (supernatant), no particles larger than 500 nm were visible by SEM (**Fig. 2b**), showing that the separation procedure had been successful. Most of the particles were in the size range of 90 to 200 nm, which was found suitable for subsequent AF4-MALS analysis. Furthermore, this size range included particles within 1 to 100 nm, which was relevant in the context of evaluating a method for detecting nanoplastics.

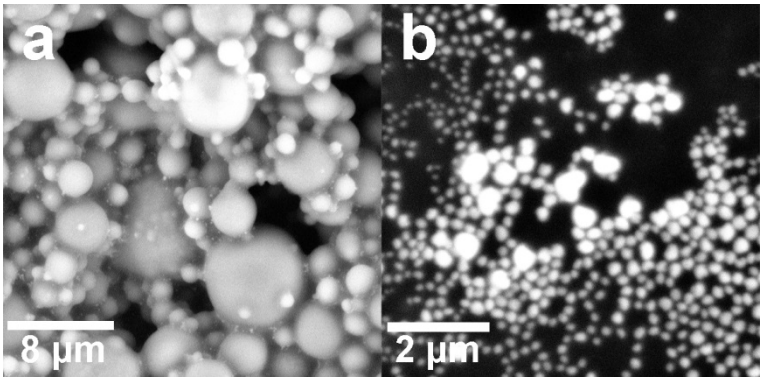


Fig. 2 SEM images of the PE sample: (A) original sample of PE particles in ethanol (10 mg mL^{-1}) (9400x original magnification); (B) isolated lower-size fraction of PE particles after centrifugation procedure (36000x original magnification).

The AF4-LS analytical procedure that was developed for PSNPs was then applied for analyzing fish that had been spiked with the lower size fraction of PE particles. In contrast to the previous results with PSNPs, there was no AF4-LS elution peak obtained in the analysis of the pure pristine PE particles (data not shown). Furthermore, there was also no elution peak observed for the fish spiked with PE particles (data not shown), showing that no PE particles could be detected even in the presence of fish digest. These results indicated that the carrier liquid (0.47 mM $NaHCO_3$ buffer) was not suitable for the analysis of the PE particles. In contrast to the PSNPs, the PE particles were not previously stabilized in aqueous solution by means of a surfactant, and might have been destabilized in the carrier liquid, where no surfactant or stabilizing agent was present. In order to stabilize the hydrophobic PE during the fractionation procedure, it was decided to test a commercial surfactant mix, FL-70 as carrier liquid for AF4 separation instead of the 0.47 mM $NaHCO_3$ buffer. FL-70 is commonly used carrier liquid in field flow fractionation and has been previously used for separation of a different types of organic or carbon based particles (e.g. carbon black, latex beads/aggregates, pigments) [16]. The remaining experimental conditions were kept.

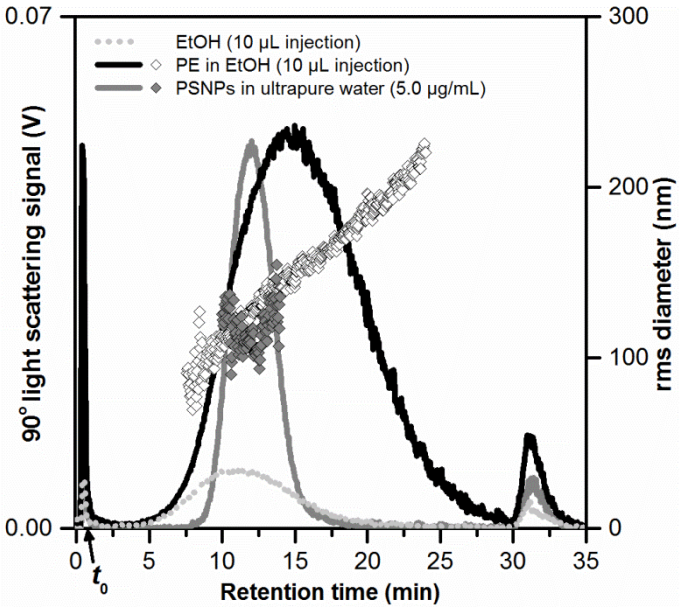


Fig. 3 AF4-LS fractograms obtained for a blank sample (ethanol injection), the lower size fraction of PE particles in ethanol and PSNPs diluted in ultrapure water using 0.025 % (v/v) FL-70 as a carrier liquid. The rms diameters determined for the PE particles and PSNPs are shown as white circles and gray diamonds,

respectively. The AF4-LS measurements for PSNPs and PE particles/EtOH were performed on separate days and using different AF4 membranes.

The AF4-LS fractograms obtained for the lower size fraction of PE particles, for a blank injection of ethanol (no particles) and for 100 nm PSNPs in ultrapure water (used as a reference) are shown in **Fig. 3** (black continuous, black dotted, and grey continuous lines, respectively). Since the mass of PE particles in the separated lower size fraction was not known, the amount of injected PE particles was adjusted experimentally until a peak with similar height to the obtained for PSNPs was achieved. A broad elution peak was obtained for the PE particles (t_r = 15.0 min), in contrast to the narrow peak obtained for the PSNPs, which eluted earlier (t_r = 12.0 min) (**Fig. 3**). This broad elution peak suggests that the PE particles have a broad size distribution, which relates well to the SEM observations that shown that the presence of PE particles with size going from 100 nm to 500 nm. The rms diameters determined for the PE particles (white circles in **Fig. 3**) increased linearly with retention time, confirming a proper elution of the particles in the AF4 channel. The determined rms diameters were in the range of 80 to 220 nm, which was also in agreement with the SEM analysis of the recovered lower-size PE fraction. A clear void peak was observed, which was probably resulting of PE particles that were not retained during separation and hence eluted at the void time t_0. A small release peak was also observed after t_r = 30 min, which corresponded to retained PE particles of larger size and that eluted when the cross-flow was set to 0 mL/min. The blank injection of ethanol (96 % purity) resulted in a broad elution peak (from t_r = 3 min to t_r = 23 min). The peak was still much smaller in magnitude compared to the one obtained for the PE sample (8.4 times lower in terms of LS peak area). Such a peak was not observed when injecting pure ultrapure water or carrier liquid into the system, indicating that ethanol caused the carry-over/release of particles from the injection system (that could be present from previous measurements). Given the higher magnitude of the PE elution peak, the influence of carry-over was not considerable for the PE concentrations that were tested in this experiment, but could hamper the detection of PE at lower concentration values. Future work is required to understand how to reduce the carry-over of particles from the AF4 and thereby be able to detect PE particles at lower concentrations with this method. The AF4-LS channel recoveries determined for the PE particles and PNSPs were satisfactory: 89 % and 77 %, respectively. The results were in agreement with the ones presented by Gigault et al. that had already shown the potential of AF4-MALS for the analysis of polydisperse PS particles (80 to 600 nm) [17]. Here we demonstrate that it is also possible to use AF4 to analyze plastic particles (and nanoplastics) of a different composition. Our AF4-MALS method (80 to 220 nm) does not cover such a wide size range as the method developed by Gigault et al. (80 to 600 nm), but could be easily adapted to cover larger particle sizes.

Since the adjusted AF4-MALS method (with 0.025 % (v/v) FL-70 as carrier liquid) was working properly for analyzing both types of particles, PSNPs or PE particles, it was decided to test the method for fish samples spiked with PSNPs or with the lower size fraction of PE particles. The obtained results demonstrated that the adjusted AF4-MALS method with 0.025 % (v/v) FL-70 as carrier liquid could be used to detect the spiked PSNPs in fish after enzymatic digestion (**Fig. 4a**). A clear AF4-LS peak was visible in the spiked fish sample (grey continuous line, retention time t_r = 10.2 min), which was not present in the blank digested fish (black dotted line). As in the case of using 0.47 mM $NaHCO_3$ as carrier liquid (Fig. 1), in 0.025% FL-70 the PSNPs were also found to elute slightly earlier when fish was present (t_r = 10.2 min compared to t_r = 12.0 min in ultrapure water). However, there was also a clear increase of background LS signal during the separation procedure for both the spiked and non-spiked fish sample. The presence of such a LS background signal indicated that there could be still some organic residues from digestion that were separated together with the particles and thus contributed to the increase in LS signal. We did not observe such a pronounced increase of the LS background signal during separation in our previous AF4-LS analysis (in 0.47 mM $NaHCO_3$) (**Fig. 1**). It is possible that the FL-70 in the carrier liquid (or one of the components present in this detergent mix) interacted with organic residues from digestion leading to the formation to the formation of a broad size range of organic structures.

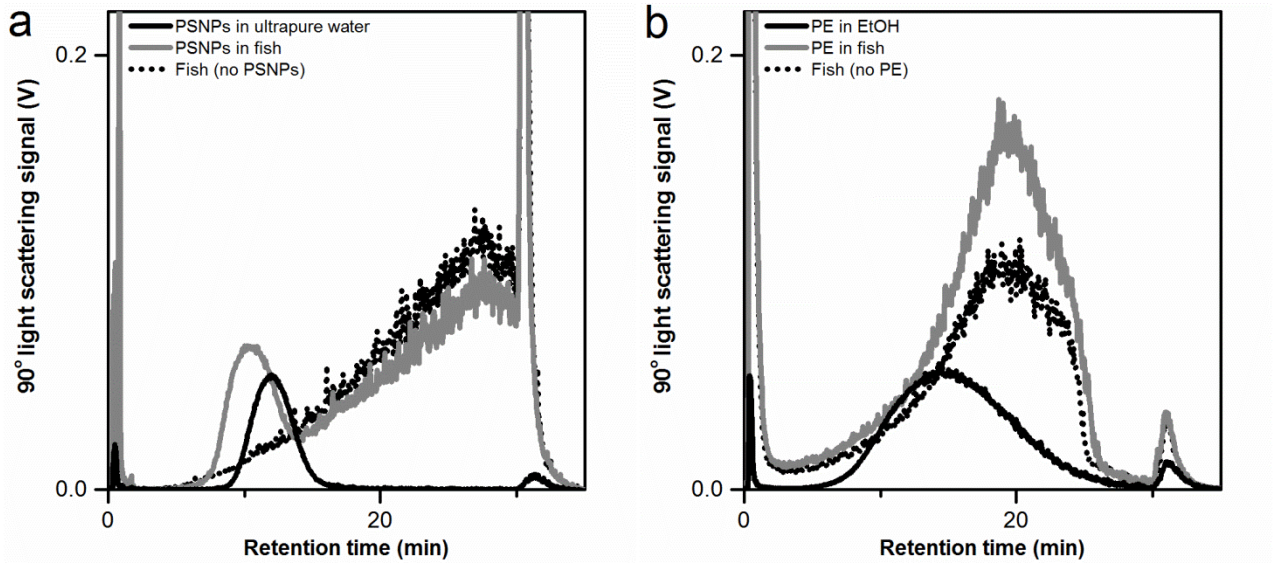


Fig. 4 AF4-LS results obtained for PSNPs (a) and PE particles (b) in fish using 0.025 % (v/v) FL-70 as a carrier liquid. For comparison, the fractograms acquired for the pristine PSNPs and PE particles (similar injection mass as spiked samples) and the non-spiked fish are shown. The injected mass of enzymatically digested fish was about five times higher in the case of spiking with PSNPs (a) than in the case of spiking with PE particles (b).

The AF4-LS fractograms obtained for the fish sample spiked with PE particles (lower size fraction) is shown in **Fig. 4b**. In contrast to the PSNPs, it was not possible to differentiate the AF4-LS fractograms obtained for the non-spiked and the PE-spiked fish sample (black dotted and gray continuous lines in **Fig. 4a**, respectively). Also in this case there was an increase of LS background signal during separation (both for non-spiked and fish spiked sample), up to a retention time of 20 to 21 min, after which the LS signal was found to drop). The AF4-LS fractogram obtained for the pristine PE particles (similar injected mass of PE as in the spiked samples) is shown as black continuous line in **Fig. 4b**. The area of the AF4-LS elution peak for the pristine PE particles was two times lower than the area of the AF4-LS elution peak for the non-spiked fish sample. Considering this, it is possible that the signal from the PE particles was simply not high enough for the detection in spiked fish samples. In these experiments it was not possible to use higher spiking masses of PE particles, as no concentrated stock suspension was available (the lower size fraction of the PE particles, as obtained from centrifugation, was spiked directly to the fish). Future work should focus on a reduction of the background signal. This could be achieved by testing other surfactants for the carrier liquid that stabilize the PE particles similarly to FL-70 but that do not interact with residues from digestion. Another alternative would be to find a different sample preparation procedure that would allow reducing the contribution of these organic residues to the LS signal.

If PE particles are not detectable after reducing the background signal, another possibility is that the PE particles were "lost" or destabilized before analysis of the fractions by AF4-MALS. The loss of PE particles by adhesion to surfaces, sedimentation, floating on the surface or similar could occur in either of the several of the steps of the procedure going from sample preparation and spiking, to enzymatic digestion and AF4 separation. The use of an alternative technique like electron microscopy will be required to investigate the presence / absence of PE particles, e.g. in the fish digest and on the AF4 membrane.

Summary and Conclusions

PSNPs could be successfully detected in a fish sample after appropriate enzymatic sample preparation and size separation with AF4. The obtained results demonstrated that combining enzymatic digestion and AF4-MALS analysis is a powerful approach for detecting nanoplastics in real food samples such as fish and

possibly other food matrices after adjusting the sample preparation procedure (using a suitable enzyme if the composition differs). Enzymatic digestion is a suitable preparation for AF4-MALS as it allows degrading the fish matrix while not affecting the main characteristics of the PSNPs (i.e. size, agglomeration state). This procedure was developed and found suitable for detection of well-dispersed spherical PSNPs, which might not be the ideal model particles for mimicking nanoplastics that present in the environment. We herein show that AF4-MALS approach could be used for analysing pristine hydrophobic PE particles after adjusting the AF4 run conditions (i.e. changing the carrier liquid from 0.47 mM $NaHCO_3$ to 0.025% FL-70). It was not possible however to detect the same PE particles when spiked to fish, in part due to the presence a higher background LS signal from organic residues during elution in these new AF4 running conditions. Our results demonstrated that an analytical method developed for a certain type of nanoplastics (such as PSNPs) may not be directly applicable to other types of nanoplastics and may need to be adjusted accordingly. It is still difficult to evaluate if the current LOD of this method is relevant in the context of real food samples, as there is no occurrence data of nanoplastics.

One limitation of the procedure relates to identification of the detected nanoplastic particles, as the LS signal is not selective to a specific type of NPs. For identification of the eluting nanoplastics, a possibility consists of off-line analysis of the collected AF4 size fractions by spectroscopy or spectrometry techniques. FTIR spectroscopy, Raman spectroscopy or py-GC/MS, which are widely used for identifying microplastics, could be used for that purpose. Other possibilities include the use of mass spectrometry techniques such as electrospray ionization mass spectrometry (ESI-MS) or matrix-assisted laser-desorption ionization coupled to time-of-flight (MALDI-TOF-MS), which could allow identifying the building blocks of nanoplastic particles. This is of course limited by the sensitivity of these techniques and since nanoplastics might be present at trace levels, an up-concentration step might be required to ensure proper detection and identification of the material in the collected fractions.

Besides for analysis of nanoplastics in food, the presented analytical methodology might also be useful for detection and quantification of nanoplastics in *in vitro* and *in vivo* studies. Fluorescently labelled particles could be used for determining particle uptake and particle concentration in cells, tissues or small organisms by AF4-MALS. A major issue in fluorescence microscopy and flow cytometry studies is the presence of an auto-fluorescence from cells/tissues. This could be avoided by separating the particles from the tissue/cells by AF4. The other advantage of our methodology compared to fluorescence microscopy, flow cytometry and other microscopy methods (FTIR or Raman confocal microscopy) is that it allows to directly access the particle size by coupling to MALS and thus to understand possible changes in particle size/agglomeration, etc. Another possibility besides fluorescent labelling would be elemental labelling of the nanoplastics. By incorporating trace elements in nanoplastic particles or using model particles with a rare element core, one could make use of the high sensitivity of ICP-MS for detection after separation by AF4.

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Complicance with Ethical Standards

Conflict of interest

The authors declare that they have no conflict of interest.

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Electronic supplementary material

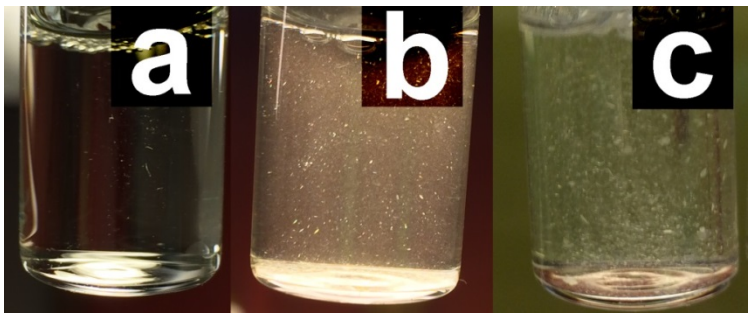

Fig. S1. Pictures of acid digests of the fish homogenates and blank PSNPs sample after dilution with 0.05 % (w/w) SDS: a) digested fish - a clear solution was visible indicating a complete digestion; b) digested fish spiked with PSNPs - a clear digest was also obtained but visible white fragments could be seen floating in solution; c) blank digested solution containing only PSNPs and no fish - visible fragments that were not present before digestion indicate that the acid treatment results in considerable particle agglomeration.

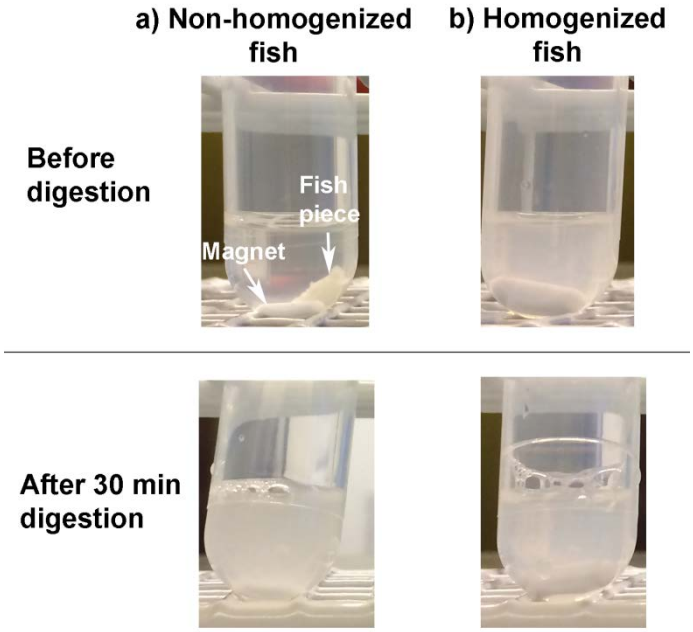

Fig. S2 Pictures of fish samples before and after 30 min enzymatic digestion with Proteinase K: a) Non-homogenized fish - a whole fish piece was used; after digestion the fish piece is completely degraded and a white clear opaque solution is obtained. b) Homogenized fish - the fish was homogenized prior to digestion; after digestion a white clear opaque solution is obtained, similar for when a whole piece was used. For all remaining experiments the fish was homogenized prior to digestion in order to ensure a proper sub-sampling of the fish.

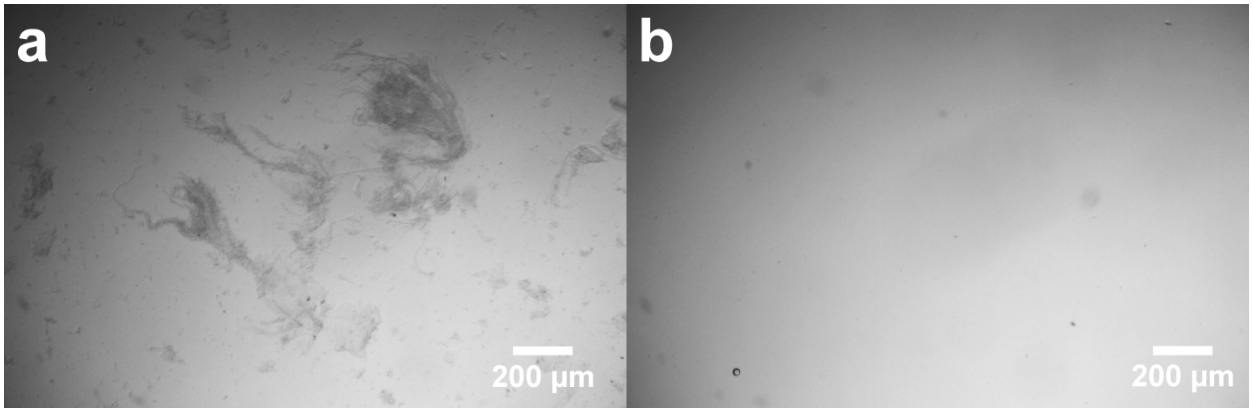

Fig. S3 Optical micrographs of a fish homogenate before (a) and after (b) enzymatic digestion (5 x magnification). Large fragments and fibers (most likely bundles of fish muscle fibers) are not visible anymore after digestion.

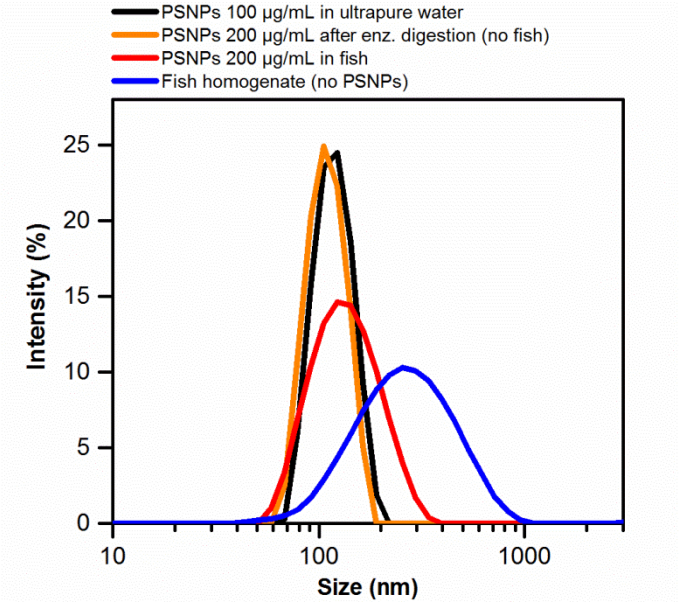

Fig. S4 Intensity based particle size distributions obtained by DLS for PSNPs in ultrapure water (black line), for a PSNPs suspension that was submitted to enzymatic digestion (orange line), for an enzymatic digest of fish containing PSNPs (red line) and for an enzymatic digest of fish not containing PSNPs (blue line). No dilution was done for the enzymatic digests.

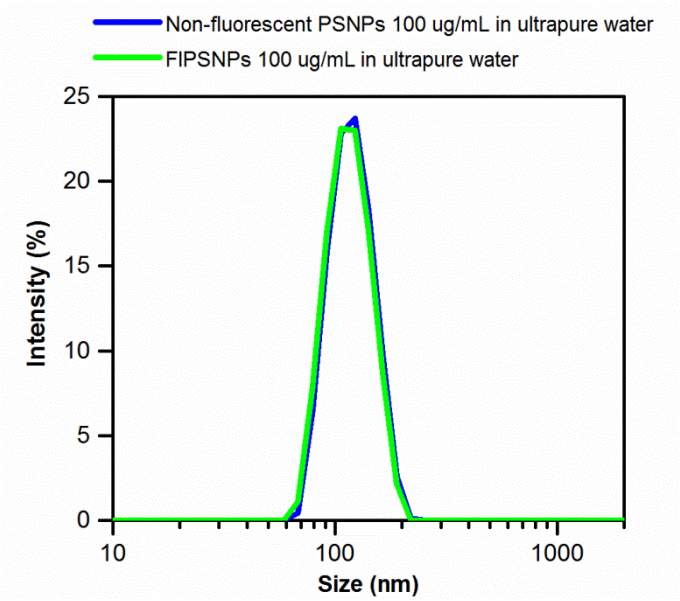

Fig. S5 Intensity based particle size distributions obtained by DLS for non-fluorescent PSNPs (blue line) and FlPSNPs in ultrapure water (black line).

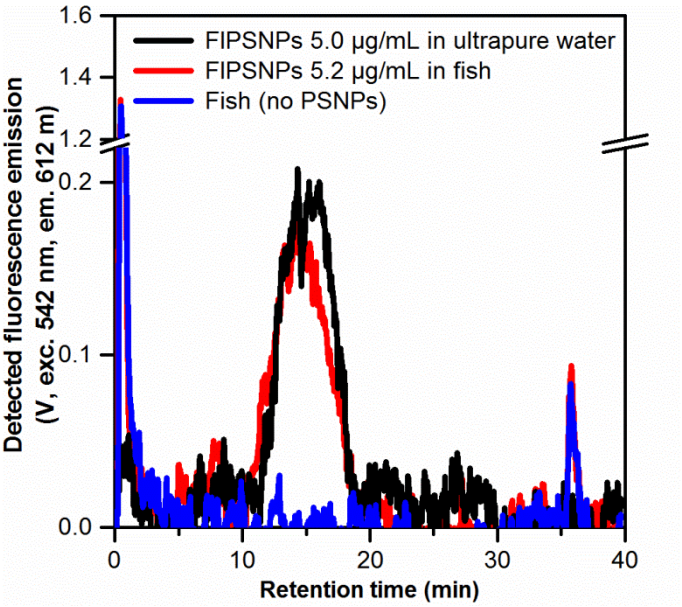

Fig. S6 AF4-fluorescence fractograms obtained for the pristine FlPSNPs in ultrapure water (black line), fish spiked with FlPSNPs (red line,), and non-spiked fish (blue line). The fish samples were enzymatically digested prior to AF4-fluorescence analysis. The fluorescence emission intensity was monitored at 612 nm, upon 542 nm excitation, to match the fluorescence properties of the FlPSNPs. The AF4-fluorescence measurements were performed using the same AF4 membrane.

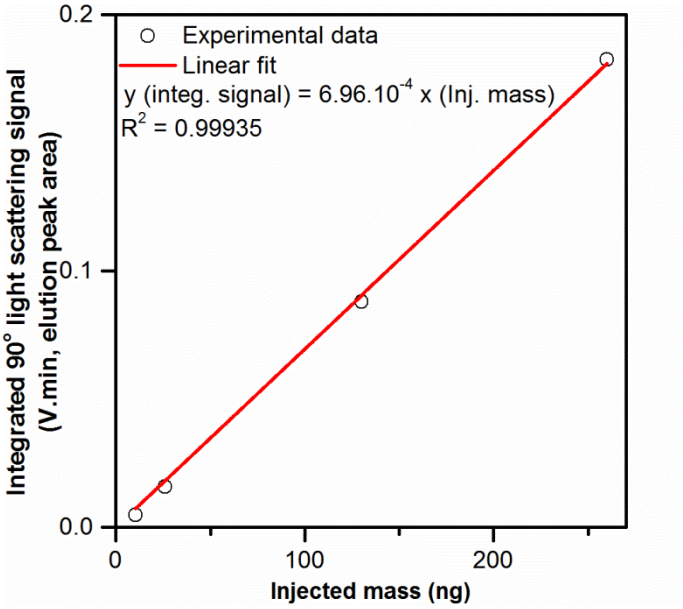

Fig. S7 Linear dependence of the AF4-LS signal (main elution peak area) on FlPSNPs injected mass for digested fish samples (overnight enzymatic digestion). The experimental data points are displayed (open circles) were fitted with a linear model (red trace).

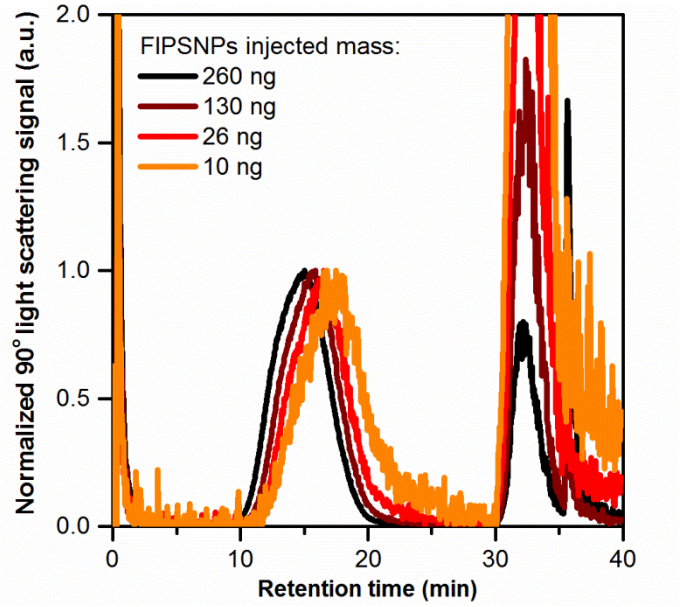

Fig. S8 AF4-LS fractograms obtained for different injected Fl. PSNPs mass values (in fish digest, overnight enzymatic digestion). The AF4-LS fractograms were normalized at the main elution peak mode for comparison. The AF4-LS measurements were performed using the same AF4 membrane.

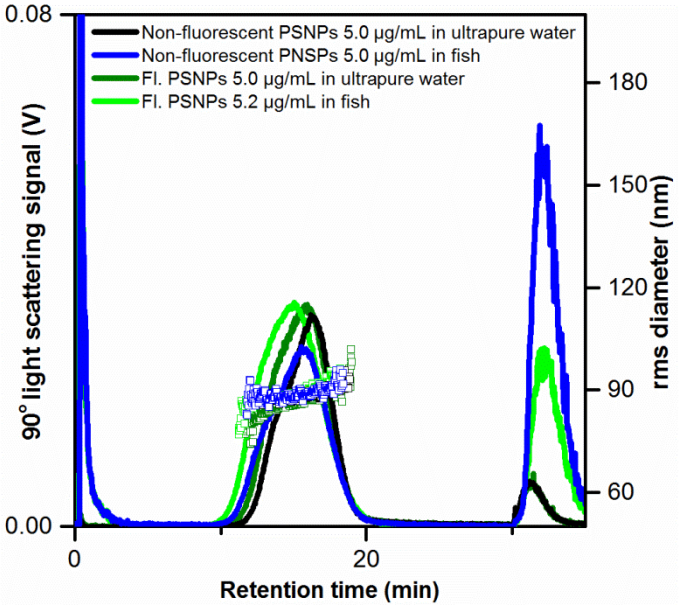

Fig. S9 AF4-LS fractograms obtained non-fluorescent PSNPs and for FlPSNPs in ultrapure water and when spiked to fish. The fish samples were enzymatically digested prior to AF4-MALS analysis. The rms diameters are shown as open squares. The AF4-LS measurements were performed using the same AF4 membrane.