Analytical approaches for the characterization and quantification of nanoparticles in food and beverages

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Graphical Abstract

1	Article Title	**Analytical approaches for the characterization and quantification of nanoparticles in food and beverages**	
2	Article Sub- Title		
3	Article Copyright - Year	**Springer-Verlag Berlin Heidelberg 2016 (This will be the copyright line in the final PDF)**	
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67	Schedule	Received	17 June 2016
68		Revised	29 August 2016
69		Accepted	14 September 2016

70 Abstract

Estimating consumer exposure to nanomaterials (NMs) in food products and predicting their toxicological properties are necessary steps in the assessment of the risks of this technology. To this end,

		analytical methods have to be available to detect, characterize and quantify NMs in food and materials related to food, e.g. food packaging and biological samples following metabolization of food. The challenge for the analytical sciences is that the characterization of NMs requires chemical as well as physical information. This article offers a comprehensive analysis of methods available for the detection and characterization of NMs in food and related products. Special attention was paid to the crucial role of sample preparation methods since these have been partially neglected in the scientific literature so far. The currently available instrumental methods are grouped as fractionation, counting and ensemble methods, and their advantages and limitations are discussed. We conclude that much progress has been made over the last 5 years but that many challenges still exist. Future perspectives and priority research needs are pointed out.
71	Keywords separated by ' - '	Nanoparticles - Nanomaterials - Emerging contaminants - Food - Analytical methods - Risk assessment
72	Foot note information	

Anal Bioanal Chem
DOI 10.1007/s00216-016-9946-5

REVIEW

Analytical approaches for the characterization and quantification of nanoparticles in food and beverages

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Received: 17 June 2016 / Revised: 29 August 2016 / Accepted: 14 September 2016

Abstract Estimating consumer exposure to nanomaterials (NMs) in food products and predicting their toxicological properties are necessary steps in the assessment of the risks of this technology. To this end, analytical methods have to be available to detect, characterize and quantify NMs in food and materials related to food, e.g. food packaging and biological samples following metabolization of food. The challenge for the analytical sciences is that the characterization of NMs requires chemical as well as physical information. This article offers a comprehensive analysis of methods available for the detection and characterization of NMs in food and related products. Special attention was paid to the crucial role of sample preparation methods since these have been partially neglected in the scientific literature so far. The currently available instrumental methods are grouped as fractionation, counting and ensemble methods, and their advantages and limitations are discussed. We conclude that much progress has been made over the last 5 years but that many challenges still exist. Future perspectives and priority research needs are pointed out.

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Keywords Nanoparticles · Nanomaterials · Emerging contaminants · Food · Analytical methods · Risk assessment

Introduction

Nanotechnology is a rapidly developing field and nanomaterials (NMs) are of significant economic interest with a global market value over 2 trillion euros in 2016 [1] and having an impact on many industries including the food industry [2]. In 2011, the European Commission (EC) released a specific recommendation on the definition of a nanomaterial: "a natural, incidental or manufactured material containing particles in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm" [3]. The EC recommendation intends to harmonize different European regulations, including REACH (Registration, Evaluation, Authorization and restriction of Chemicals) and CLP (Classification, Labelling and Packaging). In this context, analytical methods to detect, characterize and quantify NMs, as well as approaches for risk and exposure level assessment, will be required for the implementation and enforcement of such regulations [4–6]. A number of EU-funded projects are tackling this issue, including NanoDefine (http://www.nanodefine.eu/) which aims to establish analytical tools and guidance to support the implementation of the EC recommendation.

The current use of nanomaterials in the food sector can be related to three main areas: food structure, food additives and food packaging with various products for each category already available on the market. NM applications are found in the development of better tastes, enhanced flavour, texture and consistency of foodstuffs, in improved bioavailability of nutrients, in new food contact materials with particular barrier or

mechanical properties, and in nano-sensor applications for traceability and monitoring of food during transport and storage. The current available information suggests that NMs used in food and agriculture applications include both organic and inorganic materials [7]. An application area of organic NMs is the encapsulation of food additives. These so-called nutritional delivery systems or nutraceuticals are generally micelles composed of approved food-grade materials which are available as low-cost bulk ingredients [8]. The improved uptake and bioavailability thanks to encapsulation of the active ingredients has opened up a large area of applications in food and animal feed products that incorporate nano-sized vitamins, nutraceuticals, antimicrobials, antioxidants, etc. [9–12].

Inorganic NMs known to be used in food, food additives, food supplements and food packaging applications are silver, iron, calcium and magnesium, selenium, silicates and titanium dioxide [7]. Several food-grade nanoparticle products are already present on the market and thus presence of NMs in some alimentary products can be considered as being likely [13]. For instance, nanomaterials such as synthetic amorphous silica (SAS, or E551) are often added to foods that are in powder form (e.g. salt, vegetable powder, egg powder, creamer, coffee powder and so on) as an anticaking agent, thickener or carrier of flavours. While E551 is one of the most important anticaking agents, other manufactured anticaking agents include calcium silicate (E552), sodium aluminosilicate (E554), dicalcium phosphate (E341), sodium ferrocyanide (E535) and microcrystalline cellulose (E460). Titanium dioxide (TiO_2) in bulk form is approved as a food additive with number E171. It is used on a large scale as a whitener and as a colorant to impart brightness to food products, especially confectionary products. Part of the "food-grade" TiO_2 material has been shown to be nano-sized [14, 15]. Nano-silver (Ag-NM) is by volume not the most used material, but it is the fastest growing NM application in food packaging owing to its antimicrobial properties [16–19]. In the last few years there has been increasing interest in the assessment of migration of NMs from food contact materials (FCM) into food [20–22]. More recently, Ag-NMs have been studied as an alternative for the antibiotics used in poultry production [23, 24]. Many other metals in nano-sized particles are available as food or health supplements. These include nano-selenium [25], nano-calcium [26], nano-iron [27] and colloidal suspensions of metal particles, e.g. copper, gold, platinum, silver, molybdenum, palladium, titanium and zinc [28]. As a result, it is likely that consumers are exposed to such NMs on a daily basis [29, 30].

While the emerging nanotechnology holds many applications and benefits for the food sector, there are also concerns about their safety. The main concerns stem from the lack of knowledge regarding the interactions of NMs at the molecular or physiological levels, and the fact that new NMs and applications thereof are constantly being produced [7, 31–33]. In addition, the nanotechnology-derived foods are new to consumers and it remains unclear how public perception, attitudes, choice and acceptance will impact the future of such applications [34, 35]. To ensure sustainable development and use of nanotechnology, especially in the food sector, requires control and monitoring of NMs and risk assessments of their application which in turn requires information about exposure and toxicity. Even though a number of analytical methods for the detection and characterization of NMs are available [4, 36], it is clear that it is necessary to improve the analytical methods and strategies to enable risk assessments and implement future regulations [37]. Currently, risk assessments for NMs are still very challenging, and complex issues and regulations for NMs are constantly evolving [7]. Both issues impose an urgent need to develop adequate analytical methodologies for detecting and characterizing NMs. This review aims to summarize the current status of relevant analytical strategies for detection, identification and characterization of NPs in food products with particular attention to the crucial role of sample preparation strategies for achieving reliable results.

Sample preparation

In the analysis of NMs in a food sample, it has to be taken into account that these NMs are not common chemicals but highly reactive physical objects of nano-sized dimensions and characterized by a sometimes heterogeneous, evolving or vulnerable nature (i.e. in the case of core shell nanoparticles, or in the in vivo formation of a protein corona around inorganic particles). The physico-chemical properties of NMs may depend on the surrounding matrix and can change over time in response to slight perturbations of their environment. Thus, the determination of NMs in food requires sample treatment techniques that are able to extract or isolate NMs from complex matrices which may contain many more particles of a similar composition. At the same time, sample manipulation should be minimized to guarantee analytical accuracy and reduce the risks of artefacts [38]. For these reasons, the objective of sample preparation is to reduce sample complexity with good recovery rates and reproducibility, while preserving the original state and particle size distribution (PSD) of the NMs in the initial food sample [6]. The time between sample preparation and instrumental measurements and the extract storage conditions are other important parameters to be investigated in order to prevent agglomeration, de-agglomeration, dissolution and disruption phenomena, or undesired interactions with other components in the matrix extract [39]. Another point of consideration is the minimum size of the analytical sample that should be processed in order to be representative for the whole sample. Linsinger et al. concluded that the situation for NMs is comparable to that for molecules and that usually sample sizes are large enough to contain enough particles to allow approximation of the Poisson

distribution of particles by the normal distribution, which significantly reduces statistical complexity [40]. In addition, they mention that the minimum number of NMs in the subsample should be greater than 500 to limit the sampling error of the particle size distribution. Another approach to determine minimum sample size is based on Gy's equation for particle sampling [41]. Although Gy's sampling theory is hard to digest, theoretical calculations show that a sample size of only 10 mg of a sample containing 100 nm Ag nanoparticles with a particle mass concentration of 1 mg/kg is sufficient to achieve an analytical accuracy better than 10 %. In addition, the number of Ag nanoparticles in such a 10-mg sample is about 2×10^6, far more than the minimum required number of 500 mentioned by Linsinger [40]. Up to now, most of the published sample preparation procedures for nanoparticle analysis deal with aqueous environmental samples or stabilization of pure NM powders dispersed in pure water [42, 43]. However, in the last few years increasing efforts have been dedicated to more challenging solid environmental matrices, as well as to biological and food matrices [4, 44].

In the progressive preparation steps going from subsampling, particle extraction or matrix clean-up to final particle quantification, a number of quality check criteria should be in place to confirm (or at least to assess) particle size stability and recovery. In principle, stepwise sample preparation for NMs is not different from that required for classical analytes in food and beverages (Fig. 1) [45]. At first homogenization of the laboratory sample is required (step I), as is the actual extraction or isolation of NMs from the matrix (step II). Comparable with classical contaminants or residues in food, a concentration step may be required (step III). Finally, and this is different from other analytes, a stabilization of the NM suspension is often required (step IV). Step I generally consists of homogenization of the laboratory sample, which may involve manual mixing or agitation and even heating or sonication. For step II, the isolation of NPs from complex food matrices, a simple water extraction combined with sonication has often proven inadequate, resulting in low recoveries and extracts containing semi-solid matrix residues [39]. To overcome these limitations several methods are available and the choices often depend on the ruggedness of the NMs.

For inorganic NMs, sometimes called "hard" NMs, isolation is usually carried out by chemical or enzymatic digestion of the matrix. Traditional chemical digestion involves the use of strong mineral acids, often in combination with hydrogen peroxide and high temperatures [15, 45–47]. This, however, can cause the dissolution of NMs or reactions with dissolved sulfide and/or chloride species, thus losing information about their presence, size and concentration in the sample [48–50]. As a consequence, enzymatic and alkaline digestions have recently been proposed as valid alternatives for the analysis of reactive NMs in biological tissues and meat [24, 39, 50–52]. Enzymatic digestion involves NM extraction and isolation by digestion of organic matrix constituents, such as proteins or carbohydrates. For this aim, broad spectrum enzymes, as proteinase K and α-amylase have been used for the isolation of NMs in wheat, semolina, cookies, pasta [53] and chicken meat [24, 39]. For alkaline digestion, tetramethylammonium hydroxide (TMAH) is used and is able to efficiently digest soft tissue and selectively extract dissolved metals without causing the dissolution of NMs to free ions [52]. The development of an efficient matrix digestion and NM isolation procedure requires the identification and optimization of critical parameters. Parameters such as temperature and time have been demonstrated to be important for digestion efficiency and reducing the risk of NM dissolution, precipitation or aggregation [24, 39, 51]. The addition of bovine serum albumin (BSA) prior to alkaline digestion is useful to prevent NM agglomeration due to the high ionic strength of TMAH solutions [51, 54]. In addition, the material and shape of the vials and tubes, the nature and concentration of surfactants, the methods of sample agitation, i.e. vortexing, mixing, stirring or sonication, can all affect the extraction recovery [48].

The literature describes only a few extraction procedures for the isolation of NMs from the matrix. Lopez-Lorente et al.

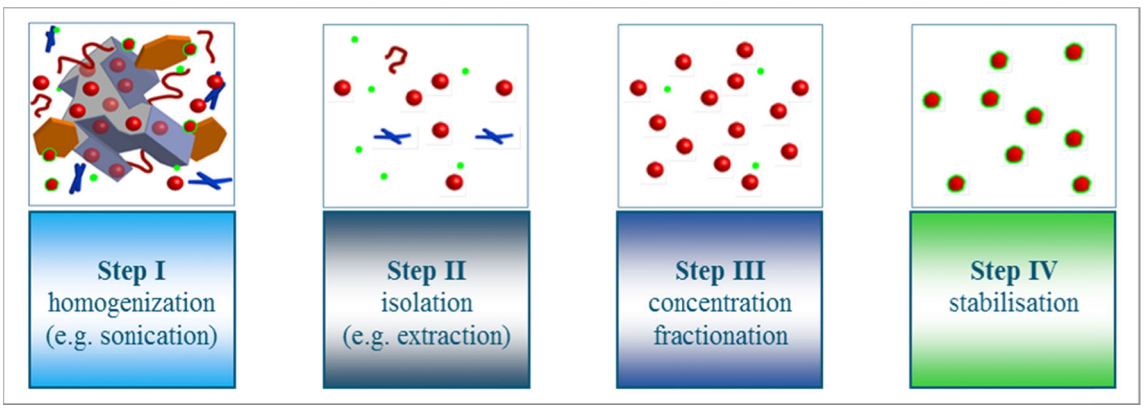

Fig. 1 Sample preparation for nanomaterials in food and consumer products. *Step I* sample homogenization (e.g. sonication). *Step II* extraction/isolation of nanomaterials. *Step III* nanomaterial concentration. *Step IV* nanomaterial stabilization

used a cationic surfactant in combination with an ionic liquid in a micro liquid–liquid extraction to isolate gold nanoparticles from water and liver [55]. In a more recent publication the same author proposed to use nanomaterial-based sorbents for the extraction of NMs of different nature [56]. The basic idea is that nanomaterials or nanostructured matter can simultaneously act as an object (the analyte) in the sample and as a (nano-)tool in different steps (sample preparation, separation and detection) of the same analytical process. An example of this is the extraction of AgNMs using a cationic surfactant in combination with sulfonated nano-cellulose as an efficient and environmental friendly dispersive micro solid-phase extraction [57]. AgNMs extracted onto the nano-cellulose sorbent are desorbed into an aqueous solution containing thiotic acid prior to capillary electrophoresis (CE) without the need for any concentration or clean-up steps.

Besides matrix complexity and the nature of the nanoparticle, the low concentration or heterogeneity of NMs in the sample can be an additional issue to be addressed, requiring prefractionation, purification and enrichment procedures such as off-line settling, centrifugation or filtration [44]. Centrifugation and ultracentrifugation techniques depend on the size, shape and density of the different sample components, whereas in membrane filtration retention and elution of an analyte depend on the size of membrane pores [58], differentiating the size range of microfiltration (100 nm–1 μm), ultrafiltration (1–100 nm) and nanofiltration (0.5–1 nm) [4, 59]. Centrifugation permits one to reach enrichment factors up to 10; however, it can also introduce the risk of particle loss due to incomplete sedimentation, or even particle alteration. Isolating nanoparticles by a filtration process is called colloidal extraction. Filtration is the most common prefractionation technique thanks to simplicity and low costs; however, it is prone to artefacts caused by membrane clogging, which decreases the effective pore size, by cake layer formation on the membrane surface during filtration and by membrane concentration polarization, thus modifying the size distribution of the samples with respect to the centrifugation [60–62]. For example, sequential filtrations of coffee creamer extract through filters with decreasing pore sizes, i.e. 5, 0.45, 0.2 and 0.1 μm, have also been investigated to achieve selectivity in the separation of the nano-silica fraction from the matrix components [63]. However, this approach resulted in losses of nano-silica during successive filtrations, probably because of nanoparticle interactions with larger components of the matrix and to poor quantitative performance of membrane filtration [64]. Cross-flow (or tangential) filtration (CFF) represents a valid alternative to dead-end filtration since it gives the advantage of reduced clogging and concentration polarization over the membrane, thanks to the tangential movement of feed flow across membrane surface [44, 65, 66]. NM enrichment can also be achieved by cloud point extraction, involving the addition of a surfactant to the sample at a concentration that exceeds the critical micelle concentration. At a temperature higher than that for a specific cloud point, the surfactant forms micelles in which non-polar substances are encapsulated. Since the density of the micelles is higher than that of water, they settle after some time, a process that is usually accelerated by centrifugation. Despite the high enrichment factors (up to 100) that can be achieved by this methodology, it is strongly influenced by matrix components and particle surface properties. Up to now, this methodology has been applied only for determination of silver NMs in water, thus allowing their separation from ionic silver [67]. The final step of sample preparation often involves particle stabilization to avoid dissolution or aggregation phenomena, in order to minimize variability effects on the final measured particle size distribution due to the sample preparation procedure. If acid digestion has been used to remove the matrix, the acid-digested sample has to be stabilized by adjustment of the pH to a range compatible with the original particle suspension. Particles may also be diluted and stabilized in a suitable dilution agent, for instance 0.01 mM sodium dodecyl sulfate (SDS) and 0.025 % (v/v) FL-70TM as detergents, able to form complexes and/or micelles, or 0.25 mM ammonium carbonate as a buffer medium in order to adjust the ionic strength and pH value [45]. As a result of all these aspects, any analytical strategy is likely to be customized on the basis of the type and nature of the NPs, the sample matrix, the instrumental separation and detection techniques and the physico-chemical properties to be assessed. Further research on the optimization of sample preparation is currently being performed within the NanoDefine project that will produce validated method and standard operating procedures (SOP) for sample preparation of certain food matrices.

The compatibility of the prepared NM extracts and requirements of the instrumental analysis must be tested beforehand. In some cases analyses of the extract with some separation and/or detection technique can be unsuccessful or even impossible. As an example, the presence of digested or partially degraded matrix can cause unresolved peaks due to non-ideal elution or shifts of retention time in the asymmetric flow field-flow fractionation (AF4) separation [39, 51]. In addition, spike experiments of TiO_2 NMs on fish tissues demonstrated that the extraction recovery depends on the type of tissue investigated (gill, liver, muscle, spleen or intestine) with lowest recovery for high-fat tissues such as liver [48]. For matrices with a high fat content, a defatting step with an organic solvent such as hexane could be included in the sample treatment procedure [53, 63]. Residues of the matrix components can be tolerated when they do not interfere with the instrumental analysis and do not change the properties and aggregation state of the particles.

In case of organic, so-called soft NMs, the sample preparation possibilities are limited since these types of nanoparticles essentially consist of micelle-like structures that break up

easily [68]. Extraction procedures to isolate intact organic NMs from a sample matrix are lacking, probably because solvent extraction generally leads to a breakup of the NM structure [69]. For instance, to isolate Coatsome A liposomes from a beverage matrix, Helsper et al. used a combination of ultrafiltration and hydrodynamic chromatography (HDC), followed by mass spectrometry-based analysis for further identification and characterization [70].

Analytical separation, detection and characterization of NMs

Analytical methods for sizing and quantification of NMs in food can be divided in three groups: fractionation, counting and ensemble methods. In the fractionation group, the most applied technique is probably AF4 which has been combined with multi-angle light scattering (MALS) and inductively coupled plasma mass spectrometry (ICP-MS) for sizing and quantification of metal and metal oxide NMs (Fig. 2). Another separation technique is hydrodynamic chromatography (HDC) which has been combined with ultraviolet (UV) and ICP-MS detectors for the detection of organic NMs and metal and metal oxide NMs. The combination of a separation technique with ICP-MS is a configuration often used, especially for metal and metal oxide NMs [71–74]. Another, less used fractionation method that will be discussed briefly is differential centrifugal sedimentation (DCS). The applications of fractionation–detection combinations for NMs in food are described in more detail in the following sections.

The best example of the counting group is electron microscopy (EM) which is recommended by the European Food Safety Agency (EFSA) for the size determination of NMs in food [75]. Normally, a prerequisite for counting methods is that the extracts need to be sufficiently clean to detect the NMs since matrix constituents that are still present in the extract will complicate the measurement or even make it impossible. Another counting technique is single particle ICP-MS (spICP-MS) (Fig. 2). Since this technique is extremely sensitive for mass (typically nanograms per litre), extract dilution before spICP-MS analysis is often required, and this dilution will contribute to the clean-up of matrix constituents [76]. Moreover since spICP-MS is element-selective, the presence of particulate matter of different chemistry will not hamper the detection, unless a clogging of the sample introduction system or the nebuliser occurs. Two counting techniques that will be discussed briefly are nanoparticle tracking analysis (NTA) and gas-phase electrophoretic mobility molecular analysis (GEMMA). The application of counting techniques for NMs in food is described in more detail in the following sections.

The third group is ensemble techniques where large numbers of NMs are measured simultaneously. Examples of that group are dynamic light scattering (DLS), particle-induced X-ray emission (PIXE), surface plasmon resonance (SPR) and coherent anti-Stokes Raman (CARS). DLS and CARS have not been used for NMs in food (DLS only in-line with a fractionation method, CARS solely for NMs in biological samples) and are therefore not discussed here. PIXE and SPR are briefly discussed in the following sections.

Table 1 lists applications of FFF and HDC in combination with UV–Vis, MALS and ICP-MS, and applications of EM and spICP-MS for the detection and characterization of NMs in food. Table 2 gives an overview of the strong and weak points of the aforementioned techniques in the detection and characterization of NMs in food.

Fractionation methods

Field-flow fractionation

In AF4 a cross-flow perpendicular to the carrier liquid is used to separate particles on the basis of their diffusion coefficient and hydrodynamic diameter [98–103]. Another field-flow fractionation technique that is used sometimes is sedimentation FFF (Sd-FFF) which uses a centrifugal field for size

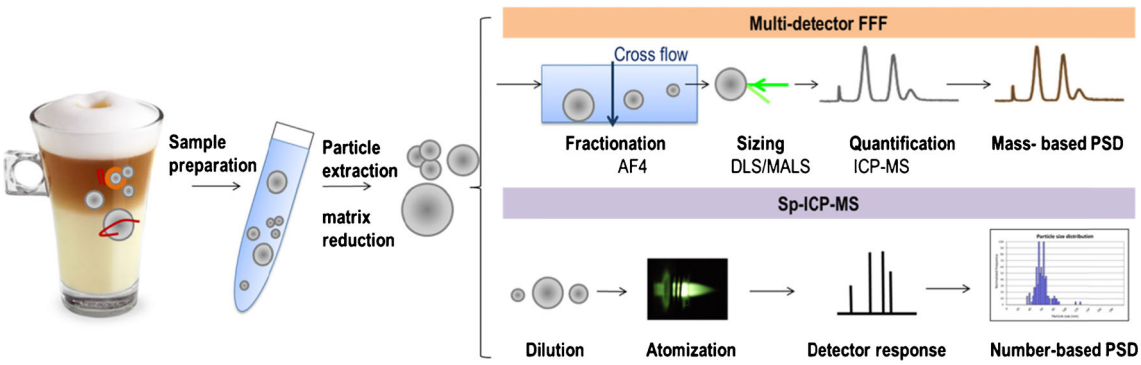

Fig. 2 Two possible analytical strategies for the sizing and quantification of NPs, indicated by *grey spheres* in the diagram, in food. *Top* AF4 with multiple detectors allows the determination of true size and a mass-based particle size distribution (PSD). *Bottom* spICP-MS allows the determination of a spherical equivalent diameter of the particle and a number-based particle size distribution

separation of particles [73]. Note that FFF methods are per se fractionation techniques able to achieve particle separation, but not to independently determine particle size. Accurate and independent size determination is only achieved by on-line coupling to a DLS detector (hydrodynamic diameter) [104], or better a multi-angle light scattering detector (MALS) able to determine the radius of gyration [79, 98, 104]. Particle size can be estimated from AF4 theory by calculating the hydrodynamic diameter based on retention time and channel dimensions, or by calibrating the separation using (certified) reference materials of known size [100]. Each approach has advantages and drawbacks. AF4 theory is based on ideal running conditions and does not account for the particle chemistry and surface charge properties that can cause large shifts in elution time [39]. The use of size calibrants, mostly polystyrene particles, is well established but also does not account for particle chemistry and surface charge properties. On-line coupling of a MALS detector allows for independent determination of the radius of gyration but is relatively expensive. Normally DLS is not suitable for samples of high complexity, because the intensity of the scattered light in the nano-range is proportional to the 6th power of the particle radius. As a result, the presence of a few large particles will easily overshadow the presence of many small particles [105, 106]. On-line coupling of FFF to a DLS detector is a convenient alternative, even if limited LOD (limit of detection) for size can hamper the sizing of the smallest particles.

Carrier flow rates in AF4 are normally 0.5–1 mL/min making AF4 compatible with MALS, US–Vis, DLS and ICP-MS detectors. UV–Vis can be helpful for detection/quantification of organic NMs and plasmonic NMs, such as nano-gold and nano-silver [100]. While inductively coupled plasma atomic emission spectroscopy (ICP-AES) [104] and atomic absorption spectroscopy (AAS) [80] have been used, on-line ICP-MS is the method of choice for element-specific detection and quantification of metal-containing NMs [79, 100]. The LOD for mass for the combination of AF4 with ICP-MS is in the order of 10 μg/L for gold and silver. For silica and titania, LODs are higher since the detection of Si and Ti is hampered by the presence of polyatomic interferences. LODs in the range of 0.16–0.3 mg/L for aqueous suspensions of silica [79] and 0.5 mg/L for titania [14] are reported. The use of collision cell technology and an MS/MS detector in ICP-MS has resulted in improved LODs for silica nanoparticles [79].

The configuration AF4-MALS-ICP-MS can be used for nano-separation, nanoparticle sizing and multi-elemental quantification. Since ICP-MS detection determines mass, the size distribution that is determined is actually a mass-based size determination. This is a drawback since the EU recommendation for a nanomaterial requires number-based size distributions; therefore, a mathematical conversion is required to translate mass-based into a number-based data [14, 73]. Although this looks straightforward, the uncertainty introduced by such a conversion results in a limitation of the lower side of the particle size distribution to 20 nm [14]. Other drawbacks of the AF4-MALS-ICP-MS combination is that it is time consuming (AF4 runtimes are typically 30–60 min), has poor dynamic size range within a run at fixed conditions and is not able to distinguish constituent particles and aggregates/agglomerates. In addition, optimization of the separation is time consuming and it often has to be tuned for different NM/matrix combinations, which means that a sound knowledge of AF4, the type of particle, its size and surface modification are required. As a consequence, AF4 is more suitable as a confirmatory technique and not as a screening technique. The multidetector FFF approach has been applied for the detection, sizing and quantification of NMs in food including silica [45] in soup, titania in food, chewing gum and toothpaste [14] and silver in chicken meat [39]. Sedimentation-FFF combined with off-line graphite furnace atomic absorption spectrometry (GFAAS) has been used for the characterization of silica particles (Aerosil300, Aerosil380, Tixosil43 and Tixosil73) used as food additives [80]. The effect of carrier pH, chemical composition and conductivity played an important role in the correct channel elution of silica. For these type of samples, preliminary preparation steps can significantly alter the particle size distribution; moreover, elution conditions including centrifugal field and carrier selection have to be finely tuned to avoid fraction losses. Contado et al. [80] achieved the best results with 0.1 % FL70 for Aerosil and could confirm the presence of a fraction of primary particles of about 10 nm and aggregates/agglomerates in the range 50–200 nm. The technique was also applied to a real cappuccino sample.

Details of key studies are summarized in Table 1, highlighting the particle–matrix addressed, type of detectors and eluents used as well as key findings. This shows that great effort is currently being put into the development and validation of general methods for preparation and analysis of nanoparticles in food, such as SiO_2 in tomato soup [45] or AgNPs in chicken meat [77] as developed in the NanoLyse project. More work is being carried out within the NanoDefine project on both preparation method and analysis by multidetector FFF approaches: further development of a generic sample preparation approach to isolate nanomaterials from food and cosmetics using a generic multistep sample preparation procedure was successfully demonstrated by Velimirovic et al. [107] for a powdered tomato soup which contains the anticaking agent SiO_2 (E551) and a sunscreen which contains TiO_2 as UV filter.

In general, the absence of official standardized protocols for a more generic FFF separation, the lack of standards certified for size and mass or number concentration, and the absence of fully inert membranes for AF4 currently represent important technical bottlenecks for the widespread use of

t1.1 **Table 1** Summary of recent papers on sizing, detection and quantification of inorganic particle in food or food-relevant matrices by means of multidetector FFF, HDC detector, spICP-MS, EM and other techniques

t1.2	Particle	Matrix	Technique	Highlights	Ref.
t1.3	Asymmetric flow field-flow fractionation (AF4) or sedimentation FFF (Sd-FFF)				
t1.4	AgNPs (PVP-stabilized) 42 ± 10 nm	Chicken meat	AF4: NH_4CO_3/pH 7: on-line MALS, US–Vis and ICP-MS	Detection and characterization of silver NPs in chicken meat by AF4 with detection by conventional or single particle ICP-MS; Non-ideal elution of NP in meat matrix on AF4	[39]
t1.5	Ag NPs 42 ± 10 nm	Chicken meat	AF4: NH_4CO_3/pH 7: on-line US–Vis and ICP-MS	In-house validation of a method for determination of silver nanoparticles in chicken meat; findings demonstrate suitability of AF4-ICP-MS for quantitative determination of AgNPs	[77]
t1.6	Ag NPs 10, 40, 60 nm	Beer	AF4: 0.01 % SDS/pH 8: on-line US–Vis and ICP-MS	Characterization and quantification of AgNPs in nutraceuticals and beverages AF4-ICP-MS; it showed large dynamic range (10–1000 μg/L) and low detection limit, 28 ng/L	[78]
t1.7	SiO_2 (Aerodisp. W7520) wide size distribution, 136 nm (D_h, DLS)	Tomato soup	AF4: 0.025 % FL70/0.25 mM NaCl: on-line MALS and ICP-MS	Description of a generic sample preparation for inorganic engineered nanoparticles in a complex matrix	[45]
t1.8	Food grade SiO_2 and TiO_2 < 20 nm	Coffee creamer	AF4: demi water: on-line MALS and ICP-MS	A systematic approach to the determination of size and concentration of SiO_2 in a real food matrix Optimization of sample preparation including matrix-to-solvent ratio, defatting with organic solvents and sonication time	[63]
t1.9	SiO_2 20, 50, 80, 100, 120, 140, 160, 180 nm and ERM-FD100	Water	AF4: 0.02 % FL-70/0.2 mM NaCl/pH 8.5: on-line MALS, US–Vis and ICP-MS/MS	Quantitative characterization of silica nanoparticles; increased sensitivity due to MS/MS	[79]
t1.10	SiO_2 (Aerosil 300, -380, Tixosil 43, -73), wide size distribution	Pure E551 and cappuccino	Sd-FFF: 0.1 % FL70: off-line GFAAS	Size characterization by Sd-FFF of silica particles used as food additives; study of effects of sample preparation on the particle size distribution	[80]
t1.11	SiO_2 20, 40, 60, 80, 100 and 150 nm	Water	AF4: 0.25 mM NH_4CO_3: on-line MALS, US–Vis and ICP-MS	Use of AF4-ICP-MS for the determination of size and concentration of SiO_2-NPs with monomodal size distributions	[81]
t1.12	TiO_2 wide distribution, 60–300 nm	Pure E171 and 24 food products	AF4: 0.02 % FL70/0.02 % NaN_3: on-line ICP-MS	All TiO_2 in food products is particulate, and about 10 % is nano-sized	
t1.13	TiO_2 wide distribution, prevalence of agglomerates/aggregates	Cosmetics/coffee cream and sugar glass	AF4: 0.2 % SDS/6 % methanol/pH 8.7: on-line US–Vis and ICP-MS	No NPs found in food, sonication time influenced PSD	[82]
t1.14	Hydrodynamic fractionation (HDC)				
t1.15	AuNPs				
t1.16	30, 60, 80 and 100 nm	Milli-Q water	HDC (PL-PSDA type 1): 10 mM SDS: on-line spICP-MS	First on-line hyphenation of HDC and spICP-MS	[83]
		Orange-flavoured beverage			[72]

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t1.18 **Table 1** (continued)

	Particle	Matrix	Technique	Highlights	Ref.
t1.17	Coatsome A, C, N -liposomes 153–205 nm		HDC (PL-PSDA type 1): 10 mM SDS: on-line UV, off-line MALDI-TOF	Characterization of three types of Coatsome liposome and validation of the HDC-UV-TOF-MS method	
t1.18	Colloidal SiO_2 Diameter 9–15 nm, length 40–100 nm	Milli-Q water	HDC (PL-PSDA type 1): 0.02 % NaN_3: on-line MALS, QUELS, VISC and DRI	Complete characterization (size, shape, compactness and particle distribution) achieved in less than 20 min	[84]
t1.19	SiO_2 (E551) 7 and 12 nm	Food products containing E551, E551 (Aerosil 200 F and 380 F)	HDC (PL-PSDA type 1): 10 mM SDS: On-line ICP-MS	Determination of nano-silica in food products with concentrations <0.1–1 mg/g and size ranges 50–200 nm	[85]
t1.20	SiO_2 10–200 nm E551/SAS and 32 nm SiO_2	Food products with E551 digested via human in vitro digestion model	HDC (PL-PSDA type 1): 10 mM SDS: on-line ICP-MS	Changes in agglomeration state of nano-sized (5–200 nm) silica in different compartments mimicking the GI system	
t1.21	Starch 57–165 nm	Waxy maize	HDC (25 × 1 cm, 5–15 μm Jordi-Gel GBR solid beads): 90 % DMSO/10 % MQ: on-line DALLS/MALLS	Ability to fractionate waxy maize amylopectins of MW > 100mln	[86]
t1.22	Single particle ICP-MS (spICP-MS)				
t1.23	AgNPs (citrate-stabilized) 60 nm	Chicken meat	spICP-MS, dwell time 3 ms	Validation of method for AgNPs in the range 5–25 mg/kg in chicken meat	
t1.24	AgNPs (PVP-stabilized) (42 ± 10 nm)	Chicken meat	spICP-MS, dwell time 3 ms	More accurate sizing than AF4-ICP-MS, results compatible with TEM	[39]
t1.25	AgNPs	Decoration pastry	spICP-MS, dwell time 3 ms	AgNPs in the size range of 10 to 50 nm found. Results comparable with EM analysis	[87]
t1.26	AgNPs 10 nm	Arabidopsis plants	spICP-MS, dwell time 50 μs	AgNPs accumulate in root tissue and tend to agglomerate, average particle size is 20 nm	[88]
t1.27	TiO_2 wide distribution 60–300 nm	Pure E171, 24 food products	spICP-MS, dwell time 3 ms	Determination of TiO_2 in 24 food products showed that ~10 % of the particles have sizes <100 nm in a number-based particle size distribution	
t1.28	Electron microscopy				
t1.29	Engineered Ag NPs 20 and 70 nm	Pear (skin and pulp)	TEM: fixation; dehydration; resin infiltration and embedding; ultrathin section cutting; staining SEM-EDS: fixation; dehydration; critical point drying; graphitization	Measured in combination with ICP-OES, Zeta-sizer	[89]
t1.30	Ag NPs 42 ± 10 nm	Chicken meat	TEM: enzymatic digestion (proteinase K); dilution	Other techniques used are AF4-ICP-MS, ICP-MS	[77]
t1.31	Ag NPs <20 nm	Silver pearls pastry decoration	TEM, STEM-EDS deposition of suspension on grid	Other technique used is spICP-MS. Size detection limit of EM better than spICP-MS	[87]
t1.32	Ag NPs 10, 40, 60 nm	Beer	TEM-EDS concentrated 10 times by evaporation; droplet on TEM grid; dried and covered with graphite coating	Concentration factor is interesting	[78]
t1.33	Ag NPs <100 nm	Apples, bread, carrot, soft cheese, meat, milk powder, orange juice, water	TEM-, SEM-EDS TEM-EDS (only orange juice): dilution in water	Also measured with ICP-MS, AAS	[90]

t1.34 **Table 1** (continued)

	Particle	Matrix	Technique	Highlights	Ref.
t1.34	Ag NPs 30–50 nm	Meat emulsion	ESEM-EDS: ashing for water containing samples TEM Dilution, homogenization, centrifugation on grids	Other technique used is EELS	[91]
t1.35	Inorganic NPs contaminants <100 nm	Bread and biscuits	ESEM-EDS Oven drying	Oven drying used as a sample preparation technique	[92]
t1.36	Metallic NPs contaminants <100 nm	Wheat seeds, semolina, wheat flour, butter cookies, pasta	SEM-EDS, ESEM-EDS treatment with TRIS buffer and heating; grinding (only for seeds); enzymatic digestion (α-amylase); defatting (only for butter cookies); filtration; filter graphitization	Also measured with ICP-MS	[53]
t1.37	NPs contaminants <10 nm	Drinking water	TEM ultracentrifugation directly on the grid	Other techniques: AFM, LIBD, STXM	[93]
t1.38	SiO_2 NPs 80 nm	Tomato soup	ASEM: deposition on the dish; dextran addition on the top of the sample	Other techniques: NTA	[94]
t1.39	Food grade SiO_2 and TiO_2 NPs <20 nm	Coffee creamer	TEM-EDS: ultracentrifugation; deposition on the copper grid TEM-EDS sample defatted with hexane; AF4 fraction centrifuged and filtered	Other techniques: AF4-ICP-MS, MALS	[63]
t1.40	Food grade-TiO_2 NPs, 40–200 nm	Chewing gum	TEM-EDS	Other techniques: XRD, DLS	[95]
t1.41	ZnO and TiO_2 NPs 20 nm and 50 nm respectively	Corn starch, yam starch, wheat flour	SEM-EDS Ashing at 750 °C for 16 h	Other techniques: ICP-OES	[96]
t1.42	TiO_2, CeO_2 and ZnO NPs <100 nm	Fish	TEM, ESEM-EDS Digestion and filtration	Other techniques: ICP-MS, ICP-OES, DLS, CARS	[44]
t1.43	Other techniques				
t1.44	AgNPs 10, 20, 60 nm	Orange juice and mussels	Capillary electrophoreses	Dispersive μSPE technique for extraction	[57]
t1.45	AgNPs	Water and fresh vegetables	Metallothionein-based surface plasmon resonance (SPR) sensor	Can provide rapid and automated analysis dedicated to environmental and food safety monitoring	[97]

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multidetector field flow fractionation as a routine analysis of NMs in food. Table 2 summarizes the pros and cons of AF4-ICP-MS in comparison to other reported methods.

Hydrodynamic chromatography

Particle size in HDC is correlated with the retention time, although interactions between the non-porous beads in the column and the analyte particles cannot be excluded. In the last few years HDC has become popular in environmental analysis to understand the behaviour, occurrence and fate of NMs [108–112]. The use of HDC for the analysis of complex samples was recently summarized by Laborda et al. [113]. Although HDC can separate a broad particle size range and can be applied in a standard liquid chromatography configuration, HDC is not very popular in the analysis of food samples. The main reason is that particle size separation in HDC is by far not as good as in AF4 [114]. Nevertheless, HDC has been used to study SiO_2 NMs in food products containing E551 [85], to investigate the fate of SiO_2 NMs after exposure to a human digestion model [30] and to separate liposome-based NMs [70]. The liposome-based NMs could not be separated with AF4 since the shear forces in the AF4 channel broke up the micelle structure of the organic NM. Table 1 summarizes the studies in which HDC coupled to different detectors was used in the analysis of food and environmental samples. Studies on both inorganic particles (SiO_2) and organic particles (liposomes and starch) have been performed; moreover, the first on-line hyphenation between HDC and spICP-MS was recently demonstrated for AuNPs, and it has been included since its application has relevance for complex matrices including food.

Differential centrifugal sedimentation

DCS, also called centrifugal liquid sedimentation (CLS), can be used for particle size characterization of materials in the range of 5 to >1000 nm. The sample is injected in the centre of a rotating disk in which a gradient of sucrose is created and in which the particles are separated before reaching the edge of the disk where a detector is located. The actual particle size is calculated from the time needed to sediment the particle and the assumed material density. DCS can separate particles that differ in diameter by as little as 5 %, including separations in complex matrices such as plasma or cell culture media. The runtime of the analysis depends on the range of sizes being analysed and the density of the particles being measured [115, 116]. For nanomaterials, analysis times are typically in the range of 15–30 min. The advantage of DCS is that in a relatively short time a high resolution is reached, and multimodal mixtures can be resolved [73]. DCS has been used for the characterization of silica nanoparticles suitable for food [117].

Counting methods

Electron microscopy

EM is the best technique to determine the shape, size and aggregation status of NPs. With a practical resolution of about 10 nm in scanning electron microscopy (SEM) and 1 nm in transmission electron microscopy (TEM), the resolution is high enough to get detailed images of NMs in food [4, 6, 118]. EM is recommended by the EFSA for the determination of particle size, shape and morphology of NMs in food, agrochemicals and food packaging and for distinguishing them from other internal components such as liposomes, micelles or crystals [75]. EMs equipped with energy dispersive X-ray spectroscopy (EDS or EDX) become even more important tools for the determination of the elemental composition of the observed NMs. Dudkiewicz et al. presented an interesting overview of EM-based methods for the characterization of NMs in food, summarizing both sample preparation for EM and imaging approaches [118, 119]. In these reviews, the authors point out that the main challenge is the sample preparation and that EM is best used as a complementary or confirmatory analysis to the analytical separation and detection techniques described in the text for these sections. In recent years, an increasing number of studies have been published concerning the determination of NMs in food matrices with EM and these are presented in Table 1. It shows that different approaches for sample preparation and different complementary techniques and typical EM problems in the characterization of NMs have been investigated in these studies.

An important aspect of EM is the limited sample volume that can be analysed. This is a consequence of the fact that SEM and TEM at high magnification are more or less surface-related analytical tools. In SEM the penetration of the X-ray beam is a few micrometres while in TEM it is only a few tens of nanometres. As a result, only a limited number of NMs can be detected or visualized in such a small volume and the limit of detection is therefore high. Random sampling and investigation of several samples are necessary to obtain representative results [119]. Automated image analysis software can improve the measurement statistics by analysing a large number of areas on the sample [53]. However, with particle concentrations at trace and ultratrace levels even this possibility runs into problems.

In conventional EM, samples are placed in a high vacuum and samples that are not electrically conductive must be coated with a conductive layer to avoid charge accumulation. However, food samples may have a considerable amount of water which means that they have to be properly fixed and dried before the analysis. If the sample morphology is expected to change as a result of the dehydration process, it is possible to encapsulate hydrated samples in thin electron-transparent membranes, or to keep them in a solid form under

cryogenic conditions. Zhang et al. demonstrated this when they studied the contamination and penetration of AgNMs in pears [89]. For the EM analysis the pear samples were initially treated with a primary fixative solution and then subjected to dehydration, followed by critical point drying using liquid carbon dioxide. This allowed them to quantify the total contamination expressed as a number-based AgNM concentration and the penetration depth of AgNMs into the fruit. While 70-nm AgNMs were stopped on the skin of the fruit, 20-nm AgNMs penetrated the fruit and diffused into the pulp.

Nowadays a good alternative to the standard high vacuum EM is low vacuum often called environmental EM (ESEM) or atmospheric EM (ASEM) in which hydrated and even liquid samples can be observed at pressures up to 6000–7000 Pa and a relative humidity up to 100 % [108, 120]. Specific sample treatments that may affect NM size and distribution in the sample matrix can thus be avoided, although the resolution of ESEM and ETEM is generally not as good as that of the high vacuum equivalent. Using ESEM, Luo et al. [120] showed that mean sizes of SiO_2 ENP in tomato soup where larger when measured with ESEM compared to TEM and FEG-SEM. This provided useful additional knowledge on the aggregation state of NMs in the food matrix. Johnston et al. exposed fish to TiO_2, CeO_2 and ZnO NPs with sizes in the 20–100 nm range [47]. Using ESEM-EDS they not only observed that different kinds of NMs concentrated in different ratios in organs but also that active mucus production in response to irritation by the exposure to NMs produced large aggregates and precipitates which increased the average size of the NMs in water which in turn decreased the bioavailability.

In most of cases, NM analysis in food by EM is used as a qualitative, and not quantitative, analysis technique. This is especially the case when it is used independently from other measurements to confirm the presence of a certain NM or when complex matrices or low particle concentrations are involved. EM is then used to support other measurements, e.g. FFF-ICP-MS [63] or AF4-ICP-MS [77], and to visually confirm the presence of NPs in the sample; however, there are exceptions. Beltrami et al., for instance, described the preparation of thin SEM-ready layers of preconcentrated samples in order to perform multiple measurements of different areas [53]. In this way they collected statistically valid data on the concentration of metal NPs in raw materials and food products, like common wheat, semolina, cookies and pasta. Similarly, Verleysen et al. described a validation method for the quantitative TEM measurement of Ag NPs in decoration pastry [87].

Single particle ICP-MS

Single particle ICP-MS (spICP-MS) has become popular for simultaneous sizing and quantifying of metal and metal oxide NMs [14, 121, 122]. In spICP-MS the number of spikes observed in the time scan is directly proportional to the particle concentration in the sample, whereas the peak height is proportional to the particle's radius to the third power. This means that a number-based particle size distribution is determined which fits well with the EC definition of a nanomaterial [3]. The particle size is calculated from the detected mass of the element that is measured assuming a certain composition and a spherical shape. However, without any a priori knowledge about a particle's composition and shape, no conclusions can be drawn about the true particle size. It is for this reason that spICP-MS is a screening method, albeit a very useful one.

An adequate time resolution and a low particle density in the sample are required to ensure that each signal originates from one particle only, hence the name *single particle* ICP-MS. While the runtime of a typical spICP-MS analysis is 1 min, the time resolution used during the run is less than 10 ms which can be handled by most standard ICP-MS systems, and more recently less than 1 ms in specialized applications on newer ICP-MS systems [123]. The short runtime makes spICP-MS analysis a much faster technique than any other for the detection of NMs. The limit of detection for mass is in the ng/L range which has the advantage that extracts can be diluted to minimize interferences from matric constituents that may be present in the extract. The size detection limit of spICP-MS depends on a number of factors including the sensitivity of the mass detector, the mass fraction of the analyte in the particles, and the background noise in the time scan [124]. For standard quadrupole ICP-MS systems the size-LOD is 10–20 nm for gold and silver, 50 nm for titania and 200 nm for silica. Calculated size-LODs for 40 elements can be found in the literature [124]. An alternative to achieve lower size-LODs is the use of a high-resolution sector-field ICP-MS which is about 10 times more sensitive than a quadrupole resulting in two times lower LODs.Table 1 lists applications of spICP-MS described in the literature for the detection of NMs in food. Of special interest is the study of silver NMs in chicken meat since it describes the validation of the complete method according to EU regulation 2002/657/EC [125]. Two inter-laboratory studies have been organised to test the performance of spICP-MS for the determination of gold and silver NMs in aqueous extracts and in digested chicken liver [94, 126]. A data evaluation tool has been developed for the calculation of particle size, particle size distribution and particle concentration from the raw spICP-MS data and is on-line available. Finally, an ISO standard is in preparation for the application of spICP-MS in aqueous extracts [127].

Recently spICP-MS has been used as a detector online with HDC as well as AF4 [83, 128]. Although data processing is still a challenge, the combination is an advantage because two independent particle sizes can be determined, one from the particle size separation (D_{HDC} or D_{AF4}), and a second from the spICP-MS analyses (D_{SP}). D_{HDC} and D_{AF4} are

t2.1 **Table 2** Synopsis of features and crucial points of AF4-ICP-MS, HDC-ICP-MS, EM-EDS and spICP-MS in the instrumental analysis of nanomaterials in food

t2.2	Performance characteristic	AF4-ICP-MS	HDC-ICP-MS	EM-EDS	sp-ICP-MS
t2.3	Determination of number-based particle size distribution as in EU recommendation for definition of nanomaterial	±	–	+	+
t2.4	Sensitivity[a] (size)	+	±	+	± (depends on element)
t2.5	Sensitivity[b] (mass)	±	±	±	+
t2.6	Discriminate between constituent particles and aggregates or agglomerates	–	–	+	–
t2.7	Discriminate between particles and ions	+	±	+	+
t2.8	Multi-elemental capability	+	+	+	± (in development)
t2.9	Capacity to size non-spherical particles	–	–	+	–
t2.10	Software for automated data processing	–	±	+	+
t2.11	Typical runtime per sample (min)	30–60	20	15	1
t2.12	Validated methods and standard operating procedures available[c]	±	–	+	±
t2.13	Specificity	+	+	–[d]	+
t2.14	Dynamic size range	±	+	–	+

– poor, ± moderate, + good

[a] Sensitivity for size to be considered within the prospective of the EU definition of nanomaterials, wherein the lowest detectable size for nanomaterials has to be 1 nm. Both AF4-ICP-MS and EM-EDS can in certain conditions achieve such sensitivity; for spICP-MS the LOD for size depends on the constituent element of the particles and the presence of interferences, with typically 10–20 nm LODs for Au and Ag, 50 nm for TiO_2 and 200 nm for SiO_2

[b] Sensitivity (for mass concentration) is considered in relation to the typical mass fraction (in weight) needed to clearly discriminate particles from the background; in the case of AF4-ICP-MS, HDC-ICP-MS, EM-EDS, reported mass LODs for mass are in the range of mg/L (for SiO_2 and TiO_2) and μg/L (for Au and Ag NPs); typical spICP-MS LODs for mass reported are in the ng/L range

[c] Some in-house validated methods have been published; refer to Table 1 for details

[d] If EDS is included in the EM analysis the – will change into +. Considering the limited resolution of EDS this is only expected for NPs with diameters >20 nm

hydrodynamic radii independent of the composition of the particle while D_{SP} is a spherical equivalent radius. If both radii are equal, the measured particle consists completely out of the measured element. If, however, $D_{SP} < D_{HDC}$ the particle consist only partly out of the measured element, as in the case of TiO_2, or the measured particle is actually an aggregate or agglomerate of the measured element. In addition, both combinations can differentiate between nanoparticles and ions, an important topic for toxicologists.

Nanoparticle tracking analysis

NTA, or nanoparticle tracking analysis is a method for sizing particles in liquids by correlating the rate of the Brownian motion to particle size [129]. The technique calculates particle size on a particle-by particle basis and allows the determination of a size distribution profile of particles with a diameter of approximately 30–1000 nm in liquid suspension. In relation to food, NTA has been used for the characterization of E551 in tomato soup [120] and the determination of Ag NMs in a chicken digest [94], and to study gold NMs in orange juice [130]. In all cases, particle size determination using NTA was reasonable (i.e. deviation less than 20 %), although the accuracy was not as good as that from EM and spICP-MS in the same samples. As with dynamic light scattering, the presence of large particles in the measurement cell easily results in overestimation from the size. The accuracy of particle concentrations determined with NTA was poor compared to the other techniques and NTA gives no information of the chemical composition of the particle.

Gas-phase electrophoretic mobility analysis

GEMMA separates single charged analytes produced by a nano-electrospray process with subsequent drying of droplets and charge conditioning in a bipolar atmosphere by a ^{210}Po α-particle source. Size separation occurs in the gas-phase employing a constant, high-laminar flow of compressed air and a tuneable electric field. By variation of the electric field strength only nanoparticles of a corresponding electrophoretic mobility diameter (EMD) are able to pass the differential mobility analyser (DMA) unit of the instrument [131]. Depending on the DMA geometry, analytes in the size range of 10–500 nm can be analysed. Subsequent detection in a condensation particle counter (CPC) is number-, not mass-based allowing the analysis of nanoparticle samples without the bias of preferential detection of high molecular mass components [132]. Additionally, as detection occurs by scattering of a focused laser beam, even single particle detection is feasible. Correlation of obtained EMD values to molecular

weights (MWs) of respective standards, allows the mass determination of analytes with unknown MW. Weiss et al. have described the separation of protein-based, gelatine nanoparticles [133].

Ensemble methods

Particle induced X-ray emission

PIXE is a technique that historically has been used to quantify trace elements in materials, like traces of metal in archaeological artefacts [134]. More recently it has been used for the detection of nanomaterials [135]. PIXE is based on exciting electronic levels of the atoms, by means of an ion beam, producing X-rays that are characteristic and proportional to elements present in the sample, thus allowing identification and quantification of the elemental composition in a single measurement. The sensitivity of PIXE is in the mg/L range and a typical runtime is 2–5 min per sample. PIXE has been used to characterize nanoparticles in rat lungs and faeces [136]. In another study Lozano et al. used PIXE to quantify dispersions of silica and silver NMs in coffee, milk and water. Since PIXE gives no information about size, size analysis was performed using DCS [137].

Surface plasmon resonance

SPR has become a well-acknowledged screening tool in the last decade that provides real-time and automated analysis with relatively high capacity [138]. Incorporation of the biological recognition elements onto the sensor surface allows detection of potentially biologically active compounds. For instance, the detection of bioavailable heavy metals can be achieved through the use of metal binding proteins such as metallothioneins (MTs) [139]. Rebe-Raz et al. [97] showed that AgNMs can be directly detected in their intact form using hMT1A protein in combination with an SPR-based sensor. The hMT1A sensor showed sensitivity in the parts per billion range, displaying the highest sensitivity towards larger and uncoated AgNMs. Potential applications of this sensor were demonstrated by successfully detecting AgNMs in fresh vegetables and river water extracts within 10 min without the need for complex sample preparation steps [97].

Conclusions and outlook

The application of nanotechnologies in the agri-food sector is expected to increase. Current and future applications involve, among others, inorganic bulk materials with size fractions below 100 nm, nano-formulated minerals, and also organic nano-carrier systems for vitamins, antioxidants and other food supplements. A number of analytical methods have been developed that can determine nanoparticles in a food matrix; however, currently only electron microscopy is expected to be suitable for classifying nanomaterials according to the EC recommendation. Challenges that remain are (i) the complexity of the matrix; (ii) the lack of certified reference materials (both for size and mass); (iii) the scarcity of specific validated methods for NPs in food and (iv) the development of new analytical techniques and strategies.

The complexity of the food matrix, and with it the need for sample preparation procedures, is a major issue. Until now most research has been in the area of instrumental detection and characterization of nanomaterials and not in the area of sample preparation. Given the interactions between NMs and many substances in food that can alter the physico-chemical status of the NMs, more development in sample preparation methods is needed. Presently each NM/matrix combination requires its own method development and optimization; however, for the near future and the detection of "unknown" NMs, generic sample preparation procedures are urgently needed. New initiatives, like the use of "nano-tools" to extract NMs are encouraging [56, 57]. In addition, the EFSA report about the use of NMs in agriculture and food identified a trend towards more organic NMs. At the moment methods for organic NMs are virtually absent [7]. New methods to detect, characterize and quantify carbon-based NMs in food or to characterize organic coatings of inorganic NMs are needed.

While aspects such as reproducibility and comparability of NP measurements are important, presently there are only a few validated methods [24]. A prerequisite for proper method validation is the availability of reference materials. While currently only suspensions of the pure NMs are available as reference materials, a few studies have been undertaken to produce reference materials of Ag and SiO_2 NMs in food [140, 141], demonstrating that development and characterization of a reference material in a food matrix is possible and that it is feasible to assign reference values with acceptable uncertainties. More reference materials are needed urgently (at this stage certification is probably too difficult).

While there is a need for standardized methods (standardized through ISO, CEN, etc.), these are not expected to become widely available for NP in matrices as complex as food. Therefore, a way forward would be the standardization of data quality by agreeing on minimum performance requirements for analytical methods and reference standards for method validation. Approaches for the validation of analytical methods for NMs in food have been proposed and applied [24, 40]. To determine the data quality of (new) analytical methods and harmonize their results, intercomparison studies are needed. Presently two international intercomparison studies have been organized and executed, but more are needed [94, 126]. Recognized reference laboratories could have a prominent role in the organisation and implementation of the suggested measures.

JnlID 216_ArtID 9946_Proof# 1 - 22/09/2016

The currently most widespread techniques for the detection and characterization of NPs in food are EM, AF4-ICP-MS and spICP-MS. Among these spICP-MS appears to be closest to a routine application owing to its relative robustness, lower requirements for sample preparation, an increasing availability of evaluation software and an ISO technical specification describing the spICP-MS procedure. AF4 still faces serious issues in reproducibility and requires trained and experienced operators. Improvements in membrane technology may dramatically improve the situation. One of the former drawbacks of EM, high costs for operation and evaluation, are currently being overcome by the development of automated operation and image analysis techniques. In time this will render the technique accessible for a broader application range, including routine analysis. The enormous diversity of NMs with different sizes, shapes, compositions and coatings easily exceeds that of conventional chemicals. Therefore it is expected that analysis of NMs is not a question of a single analytical technique but rather a combination of multiple procedures and instrumentation, and confirmation of the measurement result by a second technique which is based on a different physical principle is recommended.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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