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7		Given Name	<b>Michele</b>
8		Suffix	
9	Corresponding Author	Organization	Barilla G. R. F.Ili SpA, Advanced Laboratory Research
10		Division	
11		Address	via Mantova 166, Parma 43122
12		e-mail	Michele.Suman@barilla.com
13		Family Name	<b>Mattarozzi</b>
14		Particle	
15		Given Name	<b>Monica</b>
16		Suffix	
17	Author	Organization	Dipartimento di Chimica, Università degli Studi di Parma
18		Division	
19		Address	Parco Area delle Scienze 17/A, Parma 43124
20		e-mail	
21		Family Name	<b>Cascio</b>
22		Particle	
23		Given Name	<b>Claudia</b>
24		Suffix	
25	Author	Organization	RIKILT Wageningen UR - Institute of Food Safety
26		Division	
27		Address	P.O. Box 230, Wageningen 6700 AE
28		e-mail	
29		Family Name	<b>Calestani</b>
30		Particle	
31	Author	Given Name	<b>Davide</b>
32		Suffix	
33		Organization	IMEM-CNR, Parco Area delle Scienze 37/A
34		Division	

35		Address	Parma 43124
36		e-mail	
37		Family Name	<b>Weigel</b>
38		Particle	
39		Given Name	<b>Stefan</b>
40		Suffix	
41		Organization	RIKILT Wageningen UR - Institute of Food Safety
42		Division	
43	Author	Address	P.O. Box 230, Wageningen 6700 AE
44		Organization	BfR – Federal Institute for Risk Assessment
45		Division	
46		Address	Max-Dohm-Str. 8-10, Berlin 10589
47		Organization	BfR – Federal Institute for Risk Assessment
48		Division	
49		Address	Max-Dohm-Str. 8-10, Berlin 10589
50		e-mail	
51		Family Name	<b>Undas</b>
52		Particle	
53		Given Name	<b>Anna</b>
54		Suffix	
55	Author	Organization	RIKILT Wageningen UR - Institute of Food Safety
56		Division	
57		Address	P.O. Box 230, Wageningen 6700 AE
58		e-mail	
59		Family Name	<b>Peters</b>
60		Particle	
61		Given Name	<b>Ruud</b>
62		Suffix	
63	Author	Organization	RIKILT Wageningen UR - Institute of Food Safety
64		Division	
65		Address	P.O. Box 230, Wageningen 6700 AE
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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.

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71 **Keywords** Nanoparticles - Nanomaterials - Emerging contaminants - Food -  
separated by ' - ' Analytical methods - Risk assessment

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72 **Foot note**  
information

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

Monica Mattarozzi<sup>1</sup> · Michele Suman<sup>2</sup> · Claudia Cascio<sup>3</sup> · Davide Calestani<sup>4</sup> · Stefan Weigel<sup>3,5</sup> · Anna Undas<sup>3</sup> · Ruud Peters<sup>3</sup>

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**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.

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

**Introduction** 34

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

✉ Michele Suman  
Michele.Suman@barilla.com

<sup>1</sup> Dipartimento di Chimica, Università degli Studi di Parma, Parco Area delle Scienze 17/A, 43124 Parma, Italy

<sup>2</sup> Barilla G. R. F.lli SpA, Advanced Laboratory Research, via Mantova 166, 43122 Parma, Italy

<sup>3</sup> RIKILT Wageningen UR - Institute of Food Safety, P.O. Box 230, 6700 AE Wageningen, The Netherlands

<sup>4</sup> IMEM-CNR, Parco Area delle Scienze 37/A, 43124 Parma, Italy

<sup>5</sup> Present address: BfR – Federal Institute for Risk Assessment, Max-Dohrn-Str. 8-10, 10589 Berlin, Germany

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

77 Inorganic NMs known to be used in food, food additives,  
78 food supplements and food packaging applications are silver,  
79 iron, calcium and magnesium, selenium, silicates and titanium  
80 dioxide [7]. Several food-grade nanoparticle products are al-  
81 ready present on the market and thus presence of NMs in some  
82 alimentary products can be considered as being likely [13].  
83 For instance, nanomaterials such as synthetic amorphous silica  
84 (SAS, or E551) are often added to foods that are in powder  
85 form (e.g. salt, vegetable powder, egg powder, creamer, coffee  
86 powder and so on) as an anticaking agent, thickener or carrier  
87 of flavours. While E551 is one of the most important anticaking  
88 agents, other manufactured anticaking agents include calcium  
89 silicate (E552), sodium aluminosilicate (E554),  
90 dicalcium phosphate (E341), sodium ferrocyanide (E535)  
91 and microcrystalline cellulose (E460). Titanium dioxide  
92 ( $\text{TiO}_2$ ) in bulk form is approved as a food additive with num-  
93 ber E171. It is used on a large scale as a whitener and as a  
94 colorant to impart brightness to food products, especially con-  
95 fectionary products. Part of the “food-grade”  $\text{TiO}_2$  material  
96 has been shown to be nano-sized [14, 15]. Nano-silver (Ag-  
97 NM) is by volume not the most used material, but it is the  
98 fastest growing NM application in food packaging owing to  
99 its antimicrobial properties [16–19]. In the last few years there  
100 has been increasing interest in the assessment of migration of  
101 NMs from food contact materials (FCM) into food [20–22].  
102 More recently, Ag-NMs have been studied as an alternative  
103 for the antibiotics used in poultry production [23, 24]. Many  
104 other metals in nano-sized particles are available as food or  
105 health supplements. These include nano-selenium [25], nano-  
106 calcium [26], nano-iron [27] and colloidal suspensions of met-  
107 al particles, e.g. copper, gold, platinum, silver, molybdenum,  
108 palladium, titanium and zinc [28]. As a result, it is likely that  
109 consumers are exposed to such NMs on a daily basis [29, 30].

110 While the emerging nanotechnology holds many applica-  
111 tions and benefits for the food sector, there are also concerns  
112 about their safety. The main concerns stem from the lack of  
113 knowledge regarding the interactions of NMs at the molecular  
114 or physiological levels, and the fact that new NMs and applica-  
115 tions thereof are constantly being produced [7, 31–33]. In  
116 addition, the nanotechnology-derived foods are new to

consumers and it remains unclear how public perception, at-  
titudes, 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 imple-  
ment future regulations [37]. Currently, risk assessments for  
NMs are still very challenging, and complex issues and regu-  
lations for NMs are constantly evolving [7]. Both issues im-  
pose an urgent need to develop adequate analytical methodol-  
ogies for detecting and characterizing NMs. This review aims  
to summarize the current status of relevant analytical strategi-  
es 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 char-  
acterized by a sometimes heterogeneous, evolving or vulner-  
able nature (i.e. in the case of core shell nanoparticles, or in the  
in vivo formation of a protein corona around inorganic parti-  
cles). The physico-chemical properties of NMs may depend  
on the surrounding matrix and can change over time in re-  
sponse 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 com-  
plex 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 objec-  
tive 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 inves-  
tigated in order to prevent agglomeration, de-agglomeration,  
dissolution and disruption phenomena, or undesired interac-  
tions 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 rep-  
resentative 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



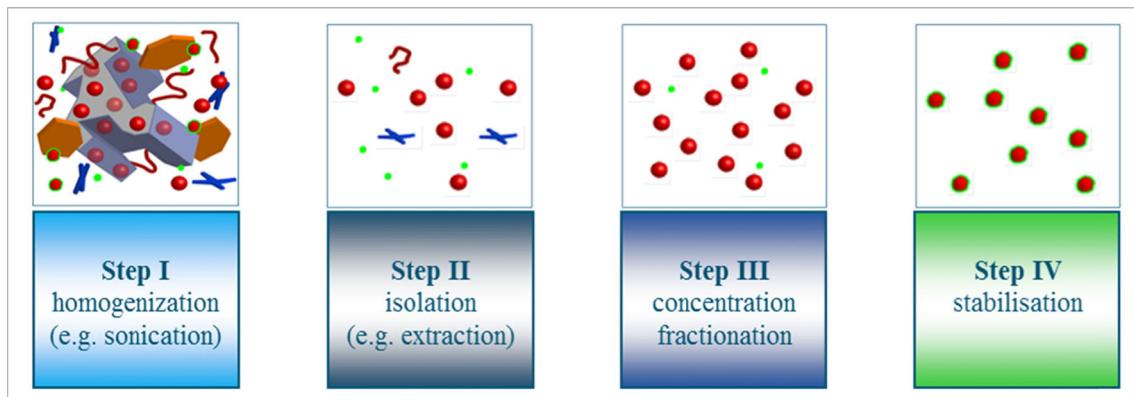
167 distribution of particles by the normal distribution, which sig- 205  
 168 nificantly reduces statistical complexity [40]. In addition, they 206  
 169 mention that the minimum number of NMs in the subsample 207  
 170 should be greater than 500 to limit the sampling error of the 208  
 171 particle size distribution. Another approach to determine min- 209  
 172 imum sample size is based on Gy's equation for particle sam- 210  
 173 pling [41]. Although Gy's sampling theory is hard to digest, 211  
 174 theoretical calculations show that a sample size of only 10 mg 212  
 175 of a sample containing 100 nm Ag nanoparticles with a parti- 213  
 176 cle mass concentration of 1 mg/kg is sufficient to achieve an 214  
 177 analytical accuracy better than 10 %. In addition, the number 215  
 178 of Ag nanoparticles in such a 10-mg sample is about  $2 \times 10^6$ , 216  
 179 far more than the minimum required number of 500 men- 217  
 180 tioned by Linsinger [40]. Up to now, most of the published 218  
 181 sample preparation procedures for nanoparticle analysis deal 219  
 182 with aqueous environmental samples or stabilization of pure 220  
 183 NM powders dispersed in pure water [42, 43]. However, in the 221  
 184 last few years increasing efforts have been dedicated to more 222  
 185 challenging solid environmental matrices, as well as to bio- 223  
 186 logical and food matrices [4, 44]. 224

187 In the progressive preparation steps going from subsam- 225  
 188 pling, particle extraction or matrix clean-up to final particle 226  
 189 quantification, a number of quality check criteria should be in 227  
 190 place to confirm (or at least to assess) particle size stability and 228  
 191 recovery. In principle, stepwise sample preparation for NMs is 229  
 192 not different from that required for classical analytes in food 230  
 193 and beverages (Fig. 1) [45]. At first homogenization of the 231  
 194 laboratory sample is required (step I), as is the actual extrac- 232  
 195 tion or isolation of NMs from the matrix (step II). Comparable 233  
 196 with classical contaminants or residues in food, a concentra- 234  
 197 tion step may be required (step III). Finally, and this is differ- 235  
 198 ent from other analytes, a stabilization of the NM suspension 236  
 199 is often required (step IV). Step I generally consists of homog- 237  
 200 enization of the laboratory sample, which may involve manual 238  
 201 mixing or agitation and even heating or sonication. For step II, 239  
 202 the isolation of NPs from complex food matrices, a simple 240  
 203 water extraction combined with sonication has often proven 241  
 204 inadequate, resulting in low recoveries and extracts containing

semi-solid matrix residues [39]. To overcome these limitations 205  
 several methods are available and the choices often depend on 206  
 the ruggedness of the NMs. 207

208 For inorganic NMs, sometimes called "hard" NMs, isola- 209  
 tion is usually carried out by chemical or enzymatic digestion 210  
 of the matrix. Traditional chemical digestion involves the use 211  
 of strong mineral acids, often in combination with hydrogen 212  
 peroxide and high temperatures [15, 45–47]. This, however, 213  
 can cause the dissolution of NMs or reactions with dissolved 214  
 sulfide and/or chloride species, thus losing information about 215  
 their presence, size and concentration in the sample [48–50]. 216  
 As a consequence, enzymatic and alkaline digestions have 217  
 recently been proposed as valid alternatives for the analysis 218  
 of reactive NMs in biological tissues and meat [24, 39, 219  
 50–52]. Enzymatic digestion involves NM extraction and isola- 220  
 tion by digestion of organic matrix constituents, such as 221  
 proteins or carbohydrates. For this aim, broad spectrum en- 222  
 zymes, as proteinase K and  $\alpha$ -amylase have been used for the 223  
 isolation of NMs in wheat, semolina, cookies, pasta [53] and 224  
 chicken meat [24, 39]. For alkaline digestion, 225  
 tetramethylammonium hydroxide (TMAH) is used and is able 226  
 to efficiently digest soft tissue and selectively extract dis- 227  
 solved metals without causing the dissolution of NMs to free 228  
 ions [52]. The development of an efficient matrix digestion 229  
 and NM isolation procedure requires the identification and 230  
 optimization of critical parameters. Parameters such as tem- 231  
 perature and time have been demonstrated to be important for 232  
 digestion efficiency and reducing the risk of NM dissolution, 233  
 precipitation or aggregation [24, 39, 51]. The addition of bo- 234  
 vine serum albumin (BSA) prior to alkaline digestion is useful 235  
 to prevent NM agglomeration due to the high ionic strength of 236  
 TMAH solutions [51, 54]. In addition, the material and shape 237  
 of the vials and tubes, the nature and concentration of surfac- 238  
 tants, the methods of sample agitation, i.e. vortexing, mixing, 239  
 stirring or sonication, can all affect the extraction recovery 240  
 [48].

241 The literature describes only a few extraction procedures 241  
 for the isolation of NMs from the matrix. Lopez-Lorente et al. 242



**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

243 used a cationic surfactant in combination with an ionic liquid  
244 in a micro liquid–liquid extraction to isolate gold nanoparti-  
245 cles from water and liver [55]. In a more recent publication the  
246 same author proposed to use nanomaterial-based sorbents for  
247 the extraction of NMs of different nature [56]. The basic idea  
248 is that nanomaterials or nanostructured matter can simulta-  
249 neously act as an object (the analyte) in the sample and as a  
250 (nano-)tool in different steps (sample preparation, separation  
251 and detection) of the same analytical process. An example of  
252 this is the extraction of AgNMs using a cationic surfactant in  
253 combination with sulfonated nano-cellulose as an efficient  
254 and environmental friendly dispersive micro solid-phase ex-  
255 traction [57]. AgNMs extracted onto the nano-cellulose sor-  
256 bent are desorbed into an aqueous solution containing thiotic  
257 acid prior to capillary electrophoresis (CE) without the need  
258 for any concentration or clean-up steps.

259 Besides matrix complexity and the nature of the nanopar-  
260 ticle, the low concentration or heterogeneity of NMs in the  
261 sample can be an additional issue to be addressed, requiring  
262 prefractionation, purification and enrichment procedures such  
263 as off-line settling, centrifugation or filtration [44].  
264 Centrifugation and ultracentrifugation techniques depend on  
265 the size, shape and density of the different sample compo-  
266 nents, whereas in membrane filtration retention and elution  
267 of an analyte depend on the size of membrane pores [58],  
268 differentiating the size range of microfiltration (100 nm–  
269 1  $\mu\text{m}$ ), ultrafiltration (1–100 nm) and nanofiltration (0.5–  
270 1 nm) [4, 59]. Centrifugation permits one to reach enrichment  
271 factors up to 10; however, it can also introduce the risk of  
272 particle loss due to incomplete sedimentation, or even particle  
273 alteration. Isolating nanoparticles by a filtration process is  
274 called colloidal extraction. Filtration is the most common  
275 prefractionation technique thanks to simplicity and low costs;  
276 however, it is prone to artefacts caused by membrane clog-  
277 ging, which decreases the effective pore size, by cake layer  
278 formation on the membrane surface during filtration and by  
279 membrane concentration polarization, thus modifying the size  
280 distribution of the samples with respect to the centrifugation  
281 [60–62]. For example, sequential filtrations of coffee creamer  
282 extract through filters with decreasing pore sizes, i.e. 5, 0.45,  
283 0.2 and 0.1  $\mu\text{m}$ , have also been investigated to achieve selec-  
284 tivity in the separation of the nano-silica fraction from the  
285 matrix components [63]. However, this approach resulted in  
286 losses of nano-silica during successive filtrations, probably  
287 because of nanoparticle interactions with larger components  
288 of the matrix and to poor quantitative performance of mem-  
289 brane filtration [64]. Cross-flow (or tangential) filtration  
290 (CFF) represents a valid alternative to dead-end filtration since  
291 it gives the advantage of reduced clogging and concentration  
292 polarization over the membrane, thanks to the tangential  
293 movement of feed flow across membrane surface [44, 65,  
294 66]. NM enrichment can also be achieved by cloud point  
295 extraction, involving the addition of a surfactant to the sample

296 at a concentration that exceeds the critical micelle concentra-  
297 tion. At a temperature higher than that for a specific cloud  
298 point, the surfactant forms micelles in which non-polar sub-  
299 stances are encapsulated. Since the density of the micelles is  
300 higher than that of water, they settle after some time, a process  
301 that is usually accelerated by centrifugation. Despite the high  
302 enrichment factors (up to 100) that can be achieved by this  
303 methodology, it is strongly influenced by matrix components  
304 and particle surface properties. Up to now, this methodology  
305 has been applied only for determination of silver NMs in  
306 water, thus allowing their separation from ionic silver [67].  
307 The final step of sample preparation often involves particle  
308 stabilization to avoid dissolution or aggregation phenomena,  
309 in order to minimize variability effects on the final measured  
310 particle size distribution due to the sample preparation proce-  
311 dure. If acid digestion has been used to remove the matrix, the  
312 acid-digested sample has to be stabilized by adjustment of the  
313 pH to a range compatible with the original particle suspension.  
314 Particles may also be diluted and stabilized in a suitable dilu-  
315 tion agent, for instance 0.01 mM sodium dodecyl sulfate  
316 (SDS) and 0.025 % (v/v) FL-70TM as detergents, able to form  
317 complexes and/or micelles, or 0.25 mM ammonium carbonate  
318 as a buffer medium in order to adjust the ionic strength and pH  
319 value [45]. As a result of all these aspects, any analytical  
320 strategy is likely to be customized on the basis of the type  
321 and nature of the NPs, the sample matrix, the instrumental  
322 separation and detection techniques and the physico-  
323 chemical properties to be assessed. Further research on the  
324 optimization of sample preparation is currently being per-  
325 formed within the NanoDefine project that will produce vali-  
326 dated method and standard operating procedures (SOP) for  
327 sample preparation of certain food matrices.

328 The compatibility of the prepared NM extracts and require-  
329 ments of the instrumental analysis must be tested beforehand.  
330 In some cases analyses of the extract with some separation  
331 and/or detection technique can be unsuccessful or even im-  
332 possible. As an example, the presence of digested or partially  
333 degraded matrix can cause unresolved peaks due to non-ideal  
334 elution or shifts of retention time in the asymmetric flow field-  
335 flow fractionation (AF4) separation [39, 51]. In addition,  
336 spike experiments of  $\text{TiO}_2$  NMs on fish tissues demonstrated  
337 that the extraction recovery depends on the type of tissue  
338 investigated (gill, liver, muscle, spleen or intestine) with low-  
339 est recovery for high-fat tissues such as liver [48]. For matrices  
340 with a high fat content, a defatting step with an organic solvent  
341 such as hexane could be included in the sample treatment  
342 procedure [53, 63]. Residues of the matrix components can  
343 be tolerated when they do not interfere with the instrumental  
344 analysis and do not change the properties and aggregation  
345 state of the particles.

346 In case of organic, so-called soft NMs, the sample prepa-  
347 ration possibilities are limited since these types of nanoparti-  
348 cles essentially consist of micelle-like structures that break up

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

357 **Analytical separation, detection and characterization**  
 358 **of NMs**

359 Analytical methods for sizing and quantification of NMs in  
 360 food can be divided in three groups: fractionation, counting  
 361 and ensemble methods. In the fractionation group, the most  
 362 applied technique is probably AF4 which has been combined  
 363 with multi-angle light scattering (MALS) and inductively  
 364 coupled plasma mass spectrometry (ICP-MS) for sizing and  
 365 quantification of metal and metal oxide NMs (Fig. 2). Another  
 366 separation technique is hydrodynamic chromatography  
 367 (HDC) which has been combined with ultraviolet (UV) and  
 368 ICP-MS detectors for the detection of organic NMs and metal  
 369 and metal oxide NMs. The combination of a separation tech-  
 370 nique with ICP-MS is a configuration often used, especially  
 371 for metal and metal oxide NMs [71–74]. Another, less used  
 372 fractionation method that will be discussed briefly is differ-  
 373 ential centrifugal sedimentation (DCS). The applications of frac-  
 374 tionation–detection combinations for NMs in food are des-  
 375 cribed in more detail in the following sections.

376 The best example of the counting group is electron micros-  
 377 copy (EM) which is recommended by the European Food  
 378 Safety Agency (EFSA) for the size determination of NMs in  
 379 food [75]. Normally, a prerequisite for counting methods is  
 380 that the extracts need to be sufficiently clean to detect the NMs  
 381 since matrix constituents that are still present in the extract will  
 382 complicate the measurement or even make it impossible.  
 383 Another counting technique is single particle ICP-MS

(spICP-MS) (Fig. 2). Since this technique is extremely sensi-  
 384 tive for mass (typically nanograms per litre), extract dilution  
 385 before spICP-MS analysis is often required, and this dilution  
 386 will contribute to the clean-up of matrix constituents [76].  
 387 Moreover since spICP-MS is element-selective, the presence  
 388 of particulate matter of different chemistry will not hamper the  
 389 detection, unless a clogging of the sample introduction system  
 390 or the nebuliser occurs. Two counting techniques that will be  
 391 discussed briefly are nanoparticle tracking analysis (NTA) and  
 392 gas-phase electrophoretic mobility molecular analysis  
 393 (GEMMA). The application of counting techniques for NMs  
 394 in food is described in more detail in the following sections.  
 395

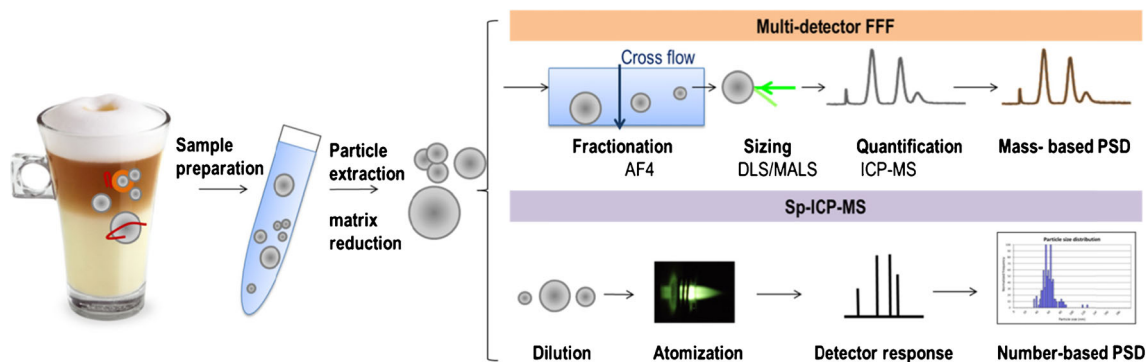
The third group is ensemble techniques where large num-  
 396 bers of NMs are measured simultaneously. Examples of that  
 397 group are dynamic light scattering (DLS), particle-induced X-  
 398 ray emission (PIXE), surface plasmon resonance (SPR) and  
 399 coherent anti-Stokes Raman (CARS). DLS and CARS have  
 400 not been used for NMs in food (DLS only in-line with a  
 401 fractionation method, CARS solely for NMs in biological  
 402 samples) and are therefore not discussed here. PIXE and  
 403 SPR are briefly discussed in the following sections.  
 404

Table 1 lists applications of FFF and HDC in combination  
 405 with UV–Vis, MALS and ICP-MS, and applications of EM  
 406 and spICP-MS for the detection and characterization of NMs  
 407 in food. Table 2 gives an overview of the strong and weak  
 408 points of the aforementioned techniques in the detection and  
 409 characterization of NMs in food.  
 410

411 **Fractionation methods**

412 **Field-flow fractionation**

In AF4 a cross-flow perpendicular to the carrier liquid is used  
 413 to separate particles on the basis of their diffusion coefficient  
 414 and hydrodynamic diameter [98–103]. Another field-flow  
 415 fractionation technique that is used sometimes is sedimenta-  
 416 tion FFF (Sd-FFF) which uses a centrifugal field for size  
 417



**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

418 separation of particles [73]. Note that FFF methods are per se  
419 fractionation techniques able to achieve particle separation,  
420 but not to independently determine particle size. Accurate  
421 and independent size determination is only achieved by on-  
422 line coupling to a DLS detector (hydrodynamic diameter)  
423 [104], or better a multi-angle light scattering detector  
424 (MALS) able to determine the radius of gyration [79, 98,  
425 104]. Particle size can be estimated from AF4 theory by cal-  
426 culating the hydrodynamic diameter based on retention time  
427 and channel dimensions, or by calibrating the separation using  
428 (certified) reference materials of known size [100]. Each ap-  
429 proach has advantages and drawbacks. AF4 theory is based on  
430 ideal running conditions and does not account for the particle  
431 chemistry and surface charge properties that can cause large  
432 shifts in elution time [39]. The use of size calibrants, mostly  
433 polystyrene particles, is well established but also does not  
434 account for particle chemistry and surface charge properties.  
435 On-line coupling of a MALS detector allows for independent  
436 determination of the radius of gyration but is relatively expen-  
437 sive. Normally DLS is not suitable for samples of high com-  
438 plexity, because the intensity of the scattered light in the nano-  
439 range is proportional to the 6th power of the particle radius. As  
440 a result, the presence of a few large particles will easily over-  
441 shadow the presence of many small particles [105, 106]. On-  
442 line coupling of FFF to a DLS detector is a convenient alter-  
443 native, even if limited LOD (limit of detection) for size can  
444 hamper the sizing of the smallest particles.

445 Carrier flow rates in AF4 are normally 0.5–1 mL/min mak-  
446 ing AF4 compatible with MALS, UV-Vis, DLS and ICP-MS  
447 detectors. UV-Vis can be helpful for detection/quantification  
448 of organic NMs and plasmonic NMs, such as nano-gold and  
449 nano-silver [100]. While inductively coupled plasma atomic  
450 emission spectroscopy (ICP-AES) [104] and atomic absorp-  
451 tion spectroscopy (AAS) [80] have been used, on-line ICP-  
452 MS is the method of choice for element-specific detection and  
453 quantification of metal-containing NMs [79, 100]. The LOD  
454 for mass for the combination of AF4 with ICP-MS is in the  
455 order of 10 µg/L for gold and silver. For silica and titania,  
456 LODs are higher since the detection of Si and Ti is hampered  
457 by the presence of polyatomic interferences. LODs in the  
458 range of 0.16–0.3 mg/L for aqueous suspensions of silica  
459 [79] and 0.5 mg/L for titania [14] are reported. The use of  
460 collision cell technology and an MS/MS detector in ICP-MS  
461 has resulted in improved LODs for silica nanoparticles [79].

462 The configuration AF4-MALS-ICP-MS can be used for  
463 nano-separation, nanoparticle sizing and multi-elemental  
464 quantification. Since ICP-MS detection determines mass,  
465 the size distribution that is determined is actually a mass-  
466 based size determination. This is a drawback since the EU  
467 recommendation for a nanomaterial requires number-based  
468 size distributions; therefore, a mathematical conversion is  
469 required to translate mass-based into a number-based data  
470 [14, 73]. Although this looks straightforward, the

471 uncertainty introduced by such a conversion results in a  
472 limitation of the lower side of the particle size distribution  
473 to 20 nm [14]. Other drawbacks of the AF4-MALS-ICP-  
474 MS combination is that it is time consuming (AF4 runtimes  
475 are typically 30–60 min), has poor dynamic size range  
476 within a run at fixed conditions and is not able to distin-  
477 guish constituent particles and aggregates/agglomerates. In  
478 addition, optimization of the separation is time consuming  
479 and it often has to be tuned for different NM/matrix com-  
480 binations, which means that a sound knowledge of AF4,  
481 the type of particle, its size and surface modification are  
482 required. As a consequence, AF4 is more suitable as a  
483 confirmatory technique and not as a screening technique.  
484 The multidetector FFF approach has been applied for the  
485 detection, sizing and quantification of NMs in food includ-  
486 ing silica [45] in soup, titania in food, chewing gum and  
487 toothpaste [14] and silver in chicken meat [39].  
488 Sedimentation-FFF combined with off-line graphite fur-  
489 nace atomic absorption spectrometry (GFAAS) has been  
490 used for the characterization of silica particles  
491 (Aerosil300, Aerosil380, Tixosil43 and Tixosil73) used  
492 as food additives [80]. The effect of carrier pH, chemical  
493 composition and conductivity played an important role in  
494 the correct channel elution of silica. For these type of sam-  
495 ples, preliminary preparation steps can significantly alter  
496 the particle size distribution; moreover, elution conditions  
497 including centrifugal field and carrier selection have to be  
498 finely tuned to avoid fraction losses. Contado et al. [80]  
499 achieved the best results with 0.1 % FL70 for Aerosil and  
500 could confirm the presence of a fraction of primary parti-  
501 cles of about 10 nm and aggregates/agglomerates in the  
502 range 50–200 nm. The technique was also applied to a real  
503 cappuccino sample.

504 Details of key studies are summarized in Table 1, highlight-  
505 ing the particle–matrix addressed, type of detectors and elu-  
506 ents used as well as key findings. This shows that great effort  
507 is currently being put into the development and validation of  
508 general methods for preparation and analysis of nanoparticles  
509 in food, such as SiO<sub>2</sub> in tomato soup [45] or AgNPs in chicken  
510 meat [77] as developed in the NanoLyse project. More work is  
511 being carried out within the NanoDefine project on both pre-  
512 paration method and analysis by multidetector FFF approaches:  
513 further development of a generic sample preparation approach  
514 to isolate nanomaterials from food and cosmetics using a ge-  
515 neric multistep sample preparation procedure was successful-  
516 ly demonstrated by Velimirovic et al. [107] for a powdered  
517 tomato soup which contains the anticaking agent SiO<sub>2</sub> (E551)  
518 and a sunscreen which contains TiO<sub>2</sub> as UV filter.

519 In general, the absence of official standardized protocols  
520 for a more generic FFF separation, the lack of standards cer-  
521 tified for size and mass or number concentration, and the  
522 absence of fully inert membranes for AF4 currently represent  
523 important technical bottlenecks for the widespread use of

**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

	Particle	Matrix	Technique	Highlights	Ref.
t1.1	Asymmetric flow field-flow fractionation (AF4) or sedimentation FFF (Sd-FFF)	Chicken meat	AF4: $\text{NH}_4\text{CO}_3/\text{pH}$ 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]
t1.2	Ag NPs $42 \pm 10$ nm	Chicken meat	AF4: $\text{NH}_4\text{CO}_3/\text{pH}$ 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]
t1.3	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 $\mu\text{g/L}$ ) and low detection limit, 28 ng/L	[78]
t1.4	$\text{SiO}_2$ (Aerodisp. W7520) wide size distribution, 136 nm ( $D_w$ , 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]
t1.5	Food grade $\text{SiO}_2$ and $\text{TiO}_2 < 20$ nm	Coffee creamer	A systematic approach to the determination of size and concentration of $\text{SiO}_2$ in a real food matrix AF4: demi water: on-line MALS and ICP-MS	Optimization of sample preparation including matrix-to-solvent ratio, defatting with organic solvents and sonication time	[63]
t1.6	$\text{SiO}_2$ 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]
t1.7	$\text{SiO}_2$ (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]
t1.8	$\text{SiO}_2$ 20, 40, 60, 80, 100 and 150 nm	Water	AF4: 0.25 mM $\text{NH}_4\text{CO}_3$ : on-line MALS, US-Vis and ICP-MS	Use of AF4-ICP-MS for the determination of size and concentration of $\text{SiO}_2$ -NPs with monomodal size distributions	[81]
t1.9	$\text{TiO}_2$ wide distribution, 60–300 nm	Pure E171 and 24 food products	AF4: 0.02 % FL70/0.02 % $\text{NaNO}_3$ : on-line ICP-MS	All $\text{TiO}_2$ in food products is particulate, and about 10 % is nano-sized	[82]
t1.10	$\text{TiO}_2$ 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]
t1.11	Hydrodynamic fractionation (HDC)	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]
t1.12	AuNPs 30, 60, 80 and 100 nm	Orange-flavoured beverage			[72]

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	[84]
t1.18 Colloidal SiO <sub>2</sub> Diameter 9–15 nm, length 40–100 nm	Milli-Q water	HDC (PL-PSDA type 1): 0.02 % Na <sub>2</sub> S <sub>2</sub> O <sub>8</sub> ; on-line MALS, QUELS, VISC and DRI	Complete characterization (size, shape, compactness and particle distribution) achieved in less than 20 min	[85]
t1.19 SiO <sub>2</sub> (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	[86]
t1.20 SiO <sub>2</sub> 10–200 nm E551/SAS and 32 nm SiO <sub>2</sub>	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	[87]
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	[88]
t1.22 Single particle ICP-MS (spICP-MS)	Chicken meat	spICP-MS; dwell time 3 ms	Validation of method for AgNPs in the range 5–25 mg/kg in chicken meat	[39]
t1.23 AgNPs (citrate-stabilized) 60 nm	Chicken meat	spICP-MS; dwell time 3 ms	More accurate sizing than AF4-ICP-MS, results compatible with TEM	[87]
t1.24 AgNPs (PVP-stabilized) (42 ± 10 nm)	Decoration pastry	spICP-MS; dwell time 3 ms	AgNPs in the size range of 10 to 50 nm found. Results comparable with EM analysis	[88]
t1.25 AgNPs	Arabidopsis plants	spICP-MS; dwell time 50 µs	AgNPs accumulate in root tissue and tend to agglomerate, average particle size is 20 nm	[89]
t1.26 AgNPs 10 nm	Pure E171, 24 food products	spICP-MS; dwell time 3 ms	Determination of TiO <sub>2</sub> in 24 food products showed that ~10 % of the particles have sizes <100 nm in a number-based particle size distribution	[89]
t1.27 TiO <sub>2</sub> wide distribution 60–300 nm				
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	[77]
t1.30 Ag NPs 42 ± 10 nm	Chicken meat	TEM: enzymatic digestion (proteinase K); dilution	Other techniques used are AF4-ICP-MS, ICP-MS	[87]
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	[78]
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	[90]
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	Other technique used is EELS	[91]
t1.35 Inorganic NPs contaminants <100 nm	Bread and biscuits	Dilution, homogenization, centrifugation on grids ESEM-EDS	Oven drying used as a sample preparation technique	[92]
t1.36 Metallic NPs contaminants <100 nm	Wheat seeds, semolina, wheat flour, butter cookies, pasta	Oven drying SEM-EDS, ESEM-EDS treatment with TRIS buffer and heating; grinding (only for seeds); enzymatic digestion ( $\alpha$ -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 <sub>2</sub> NPs 80 nm	Tomato soup ASEM	deposition on the dish; dextran addition on the top of the sample TEM-EDS: ultracentrifugation; deposition on the copper grid	Other techniques: NTA	[94]
t1.39 Food grade SiO <sub>2</sub> and TiO <sub>2</sub> NPs <20 nm	Coffee creamer	TEM-EDS sample defatted with hexane; AF4 fraction centrifuged and filtered	Other techniques: AF4-ICP-MS, MALS	[63]
t1.40 Food grade-TiO <sub>2</sub> NPs, 40–200 nm	Chewing gum	TEM-EDS	Other techniques: XRD, DLS	[95]
t1.41 ZnO and TiO <sub>2</sub> 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 <sub>2</sub> , CeO <sub>2</sub> 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	Orange juice and mussels	Capillary electrophoreses	Dispersive $\mu$ SPE technique for extraction	[57]
t1.44 AgNPs 10, 20, 60 nm	Water and fresh vegetables	Mercuric iodine-based surface plasmon resonance (SPR) sensor	Can provide rapid and automated analysis dedicated to environmental and food safety monitoring	[97]
t1.45 AgNPs				

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

527 **Hydrodynamic chromatography**

528 Particle size in HDC is correlated with the retention time,  
 529 although interactions between the non-porous beads in the  
 530 column and the analyte particles cannot be excluded. In the  
 531 last few years HDC has become popular in environmental  
 532 analysis to understand the behaviour, occurrence and fate of  
 533 NMs [108–112]. The use of HDC for the analysis of complex  
 534 samples was recently summarized by Laborda et al. [113].  
 535 Although HDC can separate a broad particle size range and  
 536 can be applied in a standard liquid chromatography configu-  
 537 ration, HDC is not very popular in the analysis of food sam-  
 538 ples. The main reason is that particle size separation in HDC is  
 539 by far not as good as in AF4 [114]. Nevertheless, HDC has  
 540 been used to study SiO<sub>2</sub> NMs in food products containing  
 541 E551 [85], to investigate the fate of SiO<sub>2</sub> NMs after exposure  
 542 to a human digestion model [30] and to separate liposome-  
 543 based NMs [70]. The liposome-based NMs could not be sep-  
 544 arated with AF4 since the shear forces in the AF4 channel  
 545 broke up the micelle structure of the organic NM. Table 1  
 546 summarizes the studies in which HDC coupled to different  
 547 detectors was used in the analysis of food and environmental  
 548 samples. Studies on both inorganic particles (SiO<sub>2</sub>) and organ-  
 549 ic particles (liposomes and starch) have been performed;  
 550 moreover, the first on-line hyphenation between HDC and  
 551 spICP-MS was recently demonstrated for AuNPs, and it has  
 552 been included since its application has relevance for complex  
 553 matrices including food.

554 **Differential centrifugal sedimentation**

555 DCS, also called centrifugal liquid sedimentation (CLS), can  
 556 be used for particle size characterization of materials in the  
 557 range of 5 to >1000 nm. The sample is injected in the centre of  
 558 a rotating disk in which a gradient of sucrose is created and in  
 559 which the particles are separated before reaching the edge of  
 560 the disk where a detector is located. The actual particle size is  
 561 calculated from the time needed to sediment the particle and  
 562 the assumed material density. DCS can separate particles that  
 563 differ in diameter by as little as 5 %, including separations in  
 564 complex matrices such as plasma or cell culture media. The  
 565 runtime of the analysis depends on the range of sizes being  
 566 analysed and the density of the particles being measured [115,  
 567 116]. For nanomaterials, analysis times are typically in the  
 568 range of 15–30 min. The advantage of DCS is that in a rela-  
 569 tively short time a high resolution is reached, and multimodal  
 570 mixtures can be resolved [73]. DCS has been used for the  
 571 characterization of silica nanoparticles suitable for food [117].

**Counting methods**

572

**Electron microscopy**

573

574 EM is the best technique to determine the shape, size and  
 575 aggregation status of NPs. With a practical resolution of about  
 576 10 nm in scanning electron microscopy (SEM) and 1 nm in  
 577 transmission electron microscopy (TEM), the resolution is  
 578 high enough to get detailed images of NMs in food [4, 6,  
 579 118]. EM is recommended by the EFSA for the determination  
 580 of particle size, shape and morphology of NMs in food, agro-  
 581 chemicals and food packaging and for distinguishing them  
 582 from other internal components such as liposomes, micelles  
 583 or crystals [75]. EMs equipped with energy dispersive X-ray  
 584 spectroscopy (EDS or EDX) become even more important  
 585 tools for the determination of the elemental composition of  
 586 the observed NMs. Dudkiewicz et al. presented an interesting  
 587 overview of EM-based methods for the characterization of  
 588 NMs in food, summarizing both sample preparation for EM  
 589 and imaging approaches [118, 119]. In these reviews, the au-  
 590 thors point out that the main challenge is the sample prepara-  
 591 tion and that EM is best used as a complementary or confir-  
 592 matory analysis to the analytical separation and detection  
 593 techniques described in the text for these sections. In recent  
 594 years, an increasing number of studies have been published  
 595 concerning the determination of NMs in food matrices with  
 596 EM and these are presented in Table 1. It shows that different  
 597 approaches for sample preparation and different complemen-  
 598 tary techniques and typical EM problems in the characteriza-  
 599 tion of NMs have been investigated in these studies.

600 An important aspect of EM is the limited sample volume  
 601 that can be analysed. This is a consequence of the fact that  
 602 SEM and TEM at high magnification are more or less surface-  
 603 related analytical tools. In SEM the penetration of the X-ray  
 604 beam is a few micrometres while in TEM it is only a few tens  
 605 of nanometres. As a result, only a limited number of NMs can  
 606 be detected or visualized in such a small volume and the limit  
 607 of detection is therefore high. Random sampling and investi-  
 608 gation of several samples are necessary to obtain representa-  
 609 tive results [119]. Automated image analysis software can  
 610 improve the measurement statistics by analysing a large num-  
 611 ber of areas on the sample [53]. However, with particle con-  
 612 centrations at trace and ultratrace levels even this possibility  
 613 runs into problems.

614 In conventional EM, samples are placed in a high vacuum  
 615 and samples that are not electrically conductive must be coat-  
 616 ed with a conductive layer to avoid charge accumulation.  
 617 However, food samples may have a considerable amount of  
 618 water which means that they have to be properly fixed and  
 619 dried before the analysis. If the sample morphology is expect-  
 620 ed to change as a result of the dehydration process, it is pos-  
 621 sible to encapsulate hydrated samples in thin electron-  
 622 transparent membranes, or to keep them in a solid form under



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

633 Nowadays a good alternative to the standard high vacuum  
 634 EM is low vacuum often called environmental EM (ESEM) or  
 635 atmospheric EM (ASEM) in which hydrated and even liquid  
 636 samples can be observed at pressures up to 6000–7000 Pa and  
 637 a relative humidity up to 100 % [108, 120]. Specific sample  
 638 treatments that may affect NM size and distribution in the  
 639 sample matrix can thus be avoided, although the resolution  
 Q6 640 of ESEM and ETEM is generally not as good as that of the  
 641 high vacuum equivalent. Using ESEM, Luo et al. [120]  
 642 showed that mean sizes of SiO<sub>2</sub> ENP in tomato soup were  
 643 larger when measured with ESEM compared to TEM and  
 644 FEG-SEM. This provided useful additional knowledge on  
 645 the aggregation state of NMs in the food matrix. Johnston  
 646 et al. exposed fish to TiO<sub>2</sub>, CeO<sub>2</sub> and ZnO NPs with sizes in  
 647 the 20–100 nm range [47]. Using ESEM-EDS they not only  
 648 observed that different kinds of NMs concentrated in different  
 649 ratios in organs but also that active mucus production in re-  
 650 sponse to irritation by the exposure to NMs produced large  
 651 aggregates and precipitates which increased the average size  
 652 of the NMs in water which in turn decreased the  
 653 bioavailability.

654 In most of cases, NM analysis in food by EM is used as a  
 655 qualitative, and not quantitative, analysis technique. This is  
 656 especially the case when it is used independently from other  
 657 measurements to confirm the presence of a certain NM or  
 658 when complex matrices or low particle concentrations are in-  
 659 volved. EM is then used to support other measurements, e.g.  
 660 FFF-ICP-MS [63] or AF4-ICP-MS [77], and to visually con-  
 661 firm the presence of NPs in the sample; however, there are  
 662 exceptions. Beltrami et al., for instance, described the prepa-  
 663 ration of thin SEM-ready layers of preconcentrated samples in  
 664 order to perform multiple measurements of different areas  
 665 [53]. In this way they collected statistically valid data on the  
 666 concentration of metal NPs in raw materials and food prod-  
 667 ucts, like common wheat, semolina, cookies and pasta.  
 668 Similarly, Verleysen et al. described a validation method for  
 669 the quantitative TEM measurement of Ag NPs in decoration  
 670 pastry [87].

### 671 Single particle ICP-MS

672 Single particle ICP-MS (spICP-MS) has become popular for  
 673 simultaneous sizing and quantifying of metal and metal oxide

NMs [14, 121, 122]. In spICP-MS the number of spikes ob- 674  
 675 served in the time scan is directly proportional to the particle  
 676 concentration in the sample, whereas the peak height is pro-  
 677 portional to the particle's radius to the third power. This means  
 678 that a number-based particle size distribution is determined  
 679 which fits well with the EC definition of a nanomaterial [3].  
 680 The particle size is calculated from the detected mass of the  
 681 element that is measured assuming a certain composition and  
 682 a spherical shape. However, without any a priori knowledge  
 683 about a particle's composition and shape, no conclusions can  
 684 be drawn about the true particle size. It is for this reason that  
 685 spICP-MS is a screening method, albeit a very useful one.

686 An adequate time resolution and a low particle density in  
 687 the sample are required to ensure that each signal originates  
 688 from one particle only, hence the name *single particle* ICP-  
 689 MS. While the runtime of a typical spICP-MS analysis is  
 690 1 min, the time resolution used during the run is less than  
 691 10 ms which can be handled by most standard ICP-MS sys-  
 692 tems, and more recently less than 1 ms in specialized applica-  
 693 tions on newer ICP-MS systems [123]. The short runtime  
 694 makes spICP-MS analysis a much faster technique than any  
 695 other for the detection of NMs. The limit of detection for mass  
 696 is in the ng/L range which has the advantage that extracts can  
 697 be diluted to minimize interferences from matrix constituents  
 698 that may be present in the extract. The size detection limit of  
 699 spICP-MS depends on a number of factors including the sensi-  
 700 tivity of the mass detector, the mass fraction of the analyte in  
 701 the particles, and the background noise in the time scan [124].  
 702 For standard quadrupole ICP-MS systems the size-LOD is  
 703 10–20 nm for gold and silver, 50 nm for titania and 200 nm  
 704 for silica. Calculated size-LODs for 40 elements can be found  
 705 in the literature [124]. An alternative to achieve lower size-  
 706 LODs is the use of a high-resolution sector-field ICP-MS  
 707 which is about 10 times more sensitive than a quadrupole  
 708 resulting in two times lower LODs. Table 1 lists applications  
 709 of spICP-MS described in the literature for the detection of  
 710 NMs in food. Of special interest is the study of silver NMs in  
 711 chicken meat since it describes the validation of the complete  
 712 method according to EU regulation 2002/657/EC [125]. Two  
 713 inter-laboratory studies have been organised to test the perfor-  
 714 mance of spICP-MS for the determination of gold and silver  
 715 NMs in aqueous extracts and in digested chicken liver [94,  
 716 126]. A data evaluation tool has been developed for the cal-  
 717 culation of particle size, particle size distribution and particle  
 718 concentration from the raw spICP-MS data and is on-line  
 719 available. Finally, an ISO standard is in preparation for the  
 720 application of spICP-MS in aqueous extracts [127].

721 Recently spICP-MS has been used as a detector online with  
 722 HDC as well as AF4 [83, 128]. Although data processing is  
 723 still a challenge, the combination is an advantage because two  
 724 independent particle sizes can be determined, one from the  
 725 particle size separation ( $D_{\text{HDC}}$  or  $D_{\text{AF4}}$ ), and a second from  
 726 the spICP-MS analyses ( $D_{\text{SP}}$ ).  $D_{\text{HDC}}$  and  $D_{\text{AF4}}$  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 <sup>a</sup> (size)	+	±	+	± (depends on element)
t2.5 Sensitivity <sup>b</sup> (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 <sup>c</sup>	±	–	+	±
t2.13 Specificity	+	+	– <sup>d</sup>	+
t2.14 Dynamic size range	±	+	–	+

– poor, ± moderate, + good

<sup>a</sup> 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<sub>2</sub> and 200 nm for SiO<sub>2</sub>

<sup>b</sup> 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<sub>2</sub> and TiO<sub>2</sub>) and µg/L (for Au and Ag NPs); typical spICP-MS LODs for mass reported are in the ng/L range

<sup>c</sup> Some in-house validated methods have been published; refer to Table 1 for details

<sup>d</sup> 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

727 hydrodynamic radii independent of the composition of the  
 728 particle while  $D_{SP}$  is a spherical equivalent radius. If both radii  
 729 are equal, the measured particle consists completely out of the  
 730 measured element. If, however,  $D_{SP} < D_{HDC}$  the particle con-  
 731 sist only partly out of the measured element, as in the case of  
 732 TiO<sub>2</sub>, or the measured particle is actually an aggregate or  
 733 agglomerate of the measured element. In addition, both com-  
 734 binations can differentiate between nanoparticles and ions, an  
 735 important topic for toxicologists.

736 **Nanoparticle tracking analysis**

737 NTA, or nanoparticle tracking analysis is a method for sizing  
 738 particles in liquids by correlating the rate of the Brownian  
 739 motion to particle size [129]. The technique calculates particle  
 740 size on a particle-by particle basis and allows the determina-  
 741 tion of a size distribution profile of particles with a diameter of  
 742 approximately 30–1000 nm in liquid suspension. In relation to  
 743 food, NTA has been used for the characterization of E551 in  
 744 tomato soup [120] and the determination of Ag NMs in a  
 745 chicken digest [94], and to study gold NMs in orange juice  
 746 [130]. In all cases, particle size determination using NTA was  
 747 reasonable (i.e. deviation less than 20 %), although the accu-  
 748 racy was not as good as that from EM and spICP-MS in the  
 749 same samples. As with dynamic light scattering, the presence

of large particles in the measurement cell easily results in 750  
 overestimation from the size. The accuracy of particle concen- 751  
 trations determined with NTA was poor compared to the other 752  
 techniques and NTA gives no information of the chemical 753  
 composition of the particle. 754

**Gas-phase electrophoretic mobility analysis** 755

GEMMA separates single charged analytes produced by a 756  
 nano-electrospray process with subsequent drying of droplets 757  
 and charge conditioning in a bipolar atmosphere by a <sup>210</sup>Po α- 758  
 particle source. Size separation occurs in the gas-phase 759  
 employing a constant, high-laminar flow of compressed air 760  
 and a tuneable electric field. By variation of the electric field 761  
 strength only nanoparticles of a corresponding electrophoretic 762  
 mobility diameter (EMD) are able to pass the differential mo- 763  
 bility analyser (DMA) unit of the instrument [131]. 764  
 Depending on the DMA geometry, analytes in the size range 765  
 of 10–500 nm can be analysed. Subsequent detection in a 766  
 condensation particle counter (CPC) is number-, not mass- 767  
 based allowing the analysis of nanoparticle samples without 768  
 the bias of preferential detection of high molecular mass com- 769  
 ponents [132]. Additionally, as detection occurs by scattering 770  
 of a focused laser beam, even single particle detection is fea- 771  
 sible. Correlation of obtained EMD values to molecular 772

773 weights (MWs) of respective standards, allows the mass de-  
774 termination of analytes with unknown MW. Weiss et al. have  
775 described the separation of protein-based, gelatine nanoparti-  
776 cles [133].

## 777 Ensemble methods

### 778 Particle induced X-ray emission

779 PIXE is a technique that historically has been used to quantify  
780 trace elements in materials, like traces of metal in archaeological  
781 artefacts [134]. More recently it has been used for the detection  
782 of nanomaterials [135]. PIXE is based on exciting electronic  
783 levels of the atoms, by means of an ion beam, producing X-  
784 rays that are characteristic and proportional to elements present  
785 in the sample, thus allowing identification and quantification of  
786 the elemental composition in a single measurement. The sensi-  
787 tivity of PIXE is in the mg/L range and a typical runtime is 2–  
788 5 min per sample. PIXE has been used to characterize nanopar-  
789 ticles in rat lungs and faeces [136]. In another study Lozano et al.  
790 used PIXE to quantify dispersions of silica and silver NMs in  
791 coffee, milk and water. Since PIXE gives no information about  
792 size, size analysis was performed using DCS [137].

### 793 Surface plasmon resonance

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

## 810 Conclusions and outlook

811 The application of nanotechnologies in the agri-food sector is  
812 expected to increase. Current and future applications involve,  
813 among others, inorganic bulk materials with size fractions  
814 below 100 nm, nano-formulated minerals, and also organic  
815 nano-carrier systems for vitamins, antioxidants and other food  
816 supplements. A number of analytical methods have been

developed that can determine nanoparticles in a food matrix; 817  
however, currently only electron microscopy is expected to be 818  
suitable for classifying nanomaterials according to the EC 819  
recommendation. Challenges that remain are (i) the complex- 820  
ity of the matrix; (ii) the lack of certified reference materials 821  
(both for size and mass); (iii) the scarcity of specific validated 822  
methods for NPs in food and (iv) the development of new 823  
analytical techniques and strategies. 824

The complexity of the food matrix, and with it the need for 825  
sample preparation procedures, is a major issue. Until now 826  
most research has been in the area of instrumental detection 827  
and characterization of nanomaterials and not in the area of 828  
sample preparation. Given the interactions between NMs and 829  
many substances in food that can alter the physico-chemical 830  
status of the NMs, more development in sample preparation 831  
methods is needed. Presently each NM/matrix combination 832  
requires its own method development and optimization; how- 833  
ever, for the near future and the detection of “unknown” NMs, 834  
generic sample preparation procedures are urgently needed. 835  
New initiatives, like the use of “nano-tools” to extract NMs 836  
are encouraging [56, 57]. In addition, the EFSA report about 837  
the use of NMs in agriculture and food identified a trend 838  
towards more organic NMs. At the moment methods for or- 839  
ganic NMs are virtually absent [7]. New methods to detect, 840  
characterize and quantify carbon-based NMs in food or to 841  
characterize organic coatings of inorganic NMs are needed. 842

While aspects such as reproducibility and comparability of 843  
NP measurements are important, presently there are only a 844  
few validated methods [24]. A prerequisite for proper method 845  
validation is the availability of reference materials. While cur- 846  
rently only suspensions of the pure NMs are available as refer- 847  
ence materials, a few studies have been undertaken to pro- 848  
duce reference materials of Ag and SiO<sub>2</sub> NMs in food [140, 849  
141], demonstrating that development and characterization of 850  
a reference material in a food matrix is possible and that it is 851  
feasible to assign reference values with acceptable uncer- 852  
tainties. More reference materials are needed urgently (at this 853  
stage certification is probably too difficult). 854

While there is a need for standardized methods (standard- 855  
ized through ISO, CEN, etc.), these are not expected to be- 856  
come widely available for NP in matrices as complex as food. 857  
Therefore, a way forward would be the standardization of data 858  
quality by agreeing on minimum performance requirements 859  
for analytical methods and reference standards for method 860  
validation. Approaches for the validation of analytical 861  
methods for NMs in food have been proposed and applied 862  
[24, 40]. To determine the data quality of (new) analytical 863  
methods and harmonize their results, intercomparison studies 864  
are needed. Presently two international intercomparison stud- 865  
ies have been organized and executed, but more are needed 866  
[94, 126]. Recognized reference laboratories could have a 867  
prominent role in the organisation and implementation of the 868  
suggested measures. 869

870 The currently most widespread techniques for the detection  
 871 and characterization of NPs in food are EM, AF4-ICP-MS and  
 872 spICP-MS. Among these spICP-MS appears to be closest to a  
 873 routine application owing to its relative robustness, lower re-  
 874 quirements for sample preparation, an increasing availability  
 875 of evaluation software and an ISO technical specification de-  
 876 scribing the spICP-MS procedure. AF4 still faces serious is-  
 877 sues in reproducibility and requires trained and experienced  
 878 operators. Improvements in membrane technology may dra-  
 879 matically improve the situation. One of the former drawbacks  
 880 of EM, high costs for operation and evaluation, are currently  
 881 being overcome by the development of automated operation  
 882 and image analysis techniques. In time this will render the  
 883 technique accessible for a broader application range, including  
 884 routine analysis. The enormous diversity of NMs with differ-  
 885 ent sizes, shapes, compositions and coatings easily exceeds  
 886 that of conventional chemicals. Therefore it is expected that  
 887 analysis of NMs is not a question of a single analytical tech-  
 888 nique but rather a combination of multiple procedures and  
 889 instrumentation, and confirmation of the measurement result  
 890 by a second technique which is based on a different physical  
 Q7 891 principle is recommended.

892 **Compliance with ethical standards**

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

Q10/Q8/Q8 897 **References**

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