# Chapter 14.

# Advanced methods for the detection of micro- and nanosystems in food

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**Abstract**

New methods are needed for the analysis of nanosystems in food products and packaging. Micro- and nanocapsules, nanohydrogels, nanoemulsions, lipid nanoparticles, micelles, metallic nanoparticles with a range of compositions and shapes must be determined in a variety of matrices. All these small entities present different interaction with their food environment and can change with time. There is no single technique that can provide all the information required therefore a range of complementary analytical approaches should be used to capture quantitative and qualitative physical and chemical properties to understand the behaviour of the nanosystems in food. This chapter addresses different stages of the analytical process illustrating recent developments made in this field. A cross-section of commonly used analytical tactics to characterise nanofood are explained, including advanced techniques that can offer valuable information, although their use is still limited for some. Sample preparation strategies and how these affect the quality parameters of measurements are discussed with special emphasis on the detection with electron microscopy and dynamic light scattering. Trends in the application of separation and detection techniques in the characterisation of nanosystems are also explained. There are important gaps of knowledge and grey areas regarding the working range of the different techniques in the characterisation of micro- and nanosystems in food. At present, feasibility studies are being carried out, which may precede a new phase for establishing guidelines and analytical protocols, and increasing automation. Exciting analytical times are foreseen.

**Keywords:** analysis of nanoparticles; electron microscopy; dynamic light scattering (DLS); Nanoparticle Tracking Analysis (NTA); hydrodynamic chromatography; field flow fractionation (FFF)

## 14.1 Introduction

The analysis of nanosystems in food products and packaging is becoming more and more necessary due to the increasing use of nanosystems in food products and their commercialization. Nanotechnology in food is being accepted once it has not been involved in controversial applications to date, in contrast to the other disruptive technologies, such as genetically modified crops, which are generating rejection by the consumers. With all, the food industry is being cautious, and not completely open to discuss its activities in this field to avoid rejection (Editorial from Nature Nanotechnology 2010) which is holding back progress. The potential risks of the intake of nanomaterials and the limited knowledge about their effects in living organisms (there are risks of cytotoxicity and systemic toxicity (Whitby and Busquets 2013; Lacey 2017)) is slowing the progress in the nanofood sector. Risk assessment should be done on case by case basis (European Commision 2013) because the behaviour of engineered nanomaterials is difficult to predict.

A limited number of commercial food products incorporating nanomaterials are in the market, and these are being successful. An example is chocolate with nano and micro $TiO_2$, which represent an estimated intake of 2-3 mg $TiO_2$/Kg (body mass) child (<10 years) in the UK (Weir et al. 2012). The Nanodatabase is an excellent on-line resource that compiles commercial products, including food, incorporating nanomaterials (DTU 2017). Analytical needs will increase in parallel with a more generalised presence of nanosystems in food and the need to enforce regulations about their use.

Defining which aspects of nanostystems in food need to be characterized requires understanding of the differential features, with respect to common ingredients, that could be related with their toxicity and enhanced activity. Among the added properties associated with

organic micro- and nanosystems are the encapsulation and transport of functional ingredients. Indeed, nanostructures can make possible the integration of high loadings of an active principle in the food, which boost the ingredient's functionality (Rasti, Erfanian, and Selamat 2017); improve the dispersion of ingredients in food media where they have some incompatibilities; or control the release of food ingredients (Comunian et al. 2017; Lei et al. 2017). Nanosystems are also useful to protect sensitive components from the surrounding environment during food processing, storage and digestion (Lei et al. 2017), or have the potential to make the products cost effective by providing greater sensorial properties with less amount of substance. The encapsulation method is chosen based on the bioactive component and matrix, and these aspects have recently been reviewed (Ângelo Cerqueira et al. 2017; Dias et al. 2017). Natural polymers (polysaccharides, proteins, lipids), which are "Generally Recognised As Safe", and combinations of them, are making up organic micro- and nanosystems in food applications. In contrast, the use of synthetic polymers for very similar purposes than in food technology, such as improved loading, bioavailability, pharmacokinetics and transport to target sites, is restricted to pharmaceutical formulations. The natural polymers in nanoingredients have the form of supramolecular structures named nanocapsules, nanohydrogels, nanoemulsions, lipid nanoparticles and micelles (de Souza Simões et al. 2017), and have been discussed elsewhere in this volume. Carbohydrates and proteins are the most commonly used encapsulating polymers. Their selection influences the size, shape and stability under different environment in the food product (Dias et al. 2017). Among polysaccharides, starch is the most widely used, alone or in combination with others, leading to nanosystems with different structures and polarities. Starch-based nanocapsules can be used to entrap macromolecules like lipids to smaller molecules such as polyphenols (F. Zhu 2017). Alginate is another carbohydrate frequently used (Comunian et al. 2017; Mokhtari, Jafari, and Assadpour 2017; Lei et al. 2017).

Besides organic nanosystems, inorganic nanostructures can be part of innovative packing materials (Luna and Vilchez 2017) and inks (Bautista et al. 2017), both sectors are having lot of strength because of their contribution to the enhancement of shelf life thanks to their capacity to reduce contact with oxygen, control bacterial growth and also can improved mechanical properties from the packaging. Inorganic nanomaterials are also present as additives in food, such as $TiO_2$ for its whitening and brightening properties (Dudefoi et al. 2017).

## 14.2 Analytical needs

The analytical approach is designed to get key information with reliability. Information from the content/function of the micro-, nanosystems is needed when developing the formulation of a new ingredient. The stability and ageing of the nanosystem within the matrix needs to be studied, and the possible toxicity of the nanofood and its compliance with the current legislation needs to be assessed. Hence, the most important properties that could be related to some sort of toxicity and thus need to be determined are particle size distribution, structure and loading capacity, which both are factors related with the enhanced properties attributed to nanomaterial. Besides these, physical characteristics of the micro- and nanosystems, evidence of their internal chemical composition, details of the interaction between the micro-, nanosystem and the active ingredient encapsulated, as well as the interaction of the nanosystem with cells/tissues, will provide important information about their toxicity and the bioavailability of the entrapped active ingredients. Finally, data regarding the chemical composition of the surface of the micro- and nanosystems can indicate their degree of hydrophilicity and interaction with the surrounding matrix. Changes in the physical and chemical characteristics of the nanosystems can alter their toxicity, and for that reason, it is important to capture all these features in the analysis.

The existence of legislation is the driving force that encourages the development of suitable analytical methods. There is no regulation devoted specifically to the inclusion of nanosystems in food, however nanosystems are included in other existing legislation or recommendations, hence nanofoods are controlled by the general food safety principles established by international regulatory organisms (Bownman and Ludlow 2017). The EU and Switzerland have additionally incorporated nano-specific provisions regarding the inclusion of nanomaterials in agri/feed/food in existing legislation (Amenta et al. 2015). There are distinct risk assessment procedures and regulations depending on whether the nanosystems are the main ingredient (classified as novel foods); when used as an additive or as part of food contact materials (Gallocchio, Belluco, and Ricci 2015). Food that newly incorporate nanoingredients require a pre-market assessment and authorisation by the European Food Safety Authority (EFSA) (Article 12 from the Regulation (EU) No1333/2008/EC, 257/2010/EC), organism that will need to measure the characteristics defining the nanoingredients including particle size and physicochemical characteristics as per their definition (European Commission, 2011). These ingredients present in the form of engineered nanomaterials will need to be clearly indicated in the list of ingredients, and their name will be followed by the word 'nano' in brackets (European Parliament, 2011). There is legislation to ensure that the substances migrating from food contact materials to food do not endanger the consumer's health or change the food properties. This is comprised in the European Regulation 1935/2004, where four food contact materials (plastics, ceramics, regenerated cellulose and active intelligent materials) have specific measures. In addition to these measures concerning the chemical composition of the packaging, the labelling of food contact materials should not be misleading, therefore, its inclusion in the label is mandatory (European Regulation 1169/2011) and analytical methodology should exist for the determination of nanosystems in that type of food product.

In food contact materials, the use of nanomaterials is regulated in plastics only, but these must have been previously specified on an authorisation list (European Parliament 2016). Regarding the implementation of the European Regulation 1935/2004 law, businesses have indicated that material-specific analytical methods to test composition, migration and risk assessment should be standardised (harmonised) and this would facilitate applying the same standards across Europe and compliance (European Parliament 2016).

There are difficulties when trying to evaluate the safety of food product regarding the presence of nanosystems. Harmonised analytical methods are not yet available today and this situation limits the reliability of the measurements and delays having appropriate contaminant limits in food. An additional difficulty found when studying the contamination of food by nanomaterials migrated from the packaging is the limited of information available regarding the nature of food contact materials (European Parliament 2016). For monitoring nanomaterials migrated to food from inks and contact materials, routine and rapid quantitative analysis of nanomaterials in the different food matrices is needed. Nanomaterials from packaging are mainly inorganic such as nano- $TiO_2$, $SiO_2$, ZnO, $Fe_3O_4$, Ag, and nanoclay but also can include organic nanomaterials such as nanocellulose in different forms (crystalline or in fibres) or nanochitosan (Bautista et al. 2017; Luna and Vilchez 2017). Nanomaterials in inks are also mainly inorganic, such as the conductive nano- Ag, or Cu, and these typical nanomaterials could be expanded to organic nanomaterials (carbon nanotubes, graphene) which are being studied (Bautista et al. 2017; Luna and Vilchez 2017). The analysis of the nanomaterials that could migrate to food has high cost in terms of access to analytical equipment, expertise and preparation, as identified by businesses (European Parliament 2016), however the expense will be at similar level than the already required routine analysis of pesticides in food carried out by solid phase extraction and chromatography coupled to mass spectrometry. Therefore, it is “affordable” and possible to have such methodology ready. Standardised methodology will be available soon given the

favourable conditions; worldwide, the food industry and relevant authorities have aligned needs (EFSA. Scientific Committee and Emerging Risks Unit 2017) and there is technical ability to make it possible. However, the development of analytical procedures aiming at guarantying the safety of consumers should be done following procedures that would allow measuring the key properties of nanomaterials related with their toxicology in complex matrices, these procedures would need to be validated before their use in routine food control analysis. There is scarcity of both suitable reference materials to be used in quality assurance and validated methods. This situation is bringing laboratories to make their own internal reference materials to assess the quality parameters of their analytical methods (Linsinger et al. 2011; Linsinger, Peters, and Weigel 2014; Dudkiewicz et al. 2015). Feasibility studies towards preparing reference materials are carried out and difficulties inherent to the changing nature of nanosystems in food are being identified. For instance, nanosystems can agglomerate once they are in the food matrix, hence the particle sizes in the food can be different to the size distribution of nanosystems in the solution used to spike the food when preparing a laboratory reference material (Grombe et al. 2015). Following, analytical approaches used for the characterisation of micro- and nanosystems in food will be discussed.

### 14.3 Sample preparation and its implications in measurements

Following the sampling stage, the analysis includes a series of steps to purifying and isolate the analytes (nanosystems in this case) from the rest of the sample. The analysis of nanosystems in food has different requirements than the analysis of traditional molecular contaminants such as pesticides. This is because particles and molecules interact very differently with the solvents and sorbents used for the purification. . However, the analysis of either nanomaterials or molecules can be affected by substances present in the sample matrix and could lead to low

accuracy in the analysis. Therefore, extraction and purification steps will improve the trueness of the measured values from nanosystems as isolated entities. In addition, precautions to preserve environmental factors which can alter the properties of the nanosystems, and maintain the interaction between the nanomaterial and the matrix will add value to the characterisation.

Treatments carried out to reduce the presence of matrix in the purified sample will define the information that can be obtained. Purification and pre-concentration of the micro- and nanosystems will be required when assessing their migration to food, or characterising nano-food, as the food samples may have high matrix content. In this scenario, complex matrices could reduce accuracy in quantitative analysis when using main quantitative techniques (i.e., Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), Liquid Chromatography-Mass Spectrometry (LC-MS), Gas Chromatography-Mass Spectrometry (GC-MS), porosimetry) or introduce artifacts in qualitative analysis (i.e., Raman spectroscopy, IR spectroscopy, Dynamic Light Scattering (DLS), UV spectroscopy, Scanning Electron Microscopy (SEM)). Particles will need to be separated and washed from the matrix, despite that the purification will alter the disposition of the nanosystems in the food environment. Keeping the nanosystems within the matrix during the analysis can be achieved by sacrificing quantitative results or using highly selective techniques (Environmental SEM (ESEM), confocal laser scanning microscopy (CLSM), X Ray Diffraction (XRD), X-Ray Photoelectron Spectroscopy (XPS)). However, there are cases where sample treatment has not been necessary despite using a technique that would commonly require working with purified samples. For instance, the effect of antioxidants in nanoform dispersed in an active coating was assessed through the oxidation degree of the surface of an active coating with IR. The assessment was carried out by comparing the ratio of the intensities of the bands corresponding to O-H stretching (3300 $cm^{-1}$), with the band from the C-O stretch (1140 $cm^{-1}$), which was assumed to remain unaltered by the presence of antioxidants (López-Córdoba et al. 2017). Lower intensity of the O-H band was

found with presence of the antioxidant. In this case purification steps were not needed, given that the active coating did not contain food matrix, and the signal studied was highly related to the effect of antioxidants,.

Sample treatment has high relevance when the procedure applied can affect the accuracy of the determination of the particle size of micro- and nanosystems. This is because particle size is one of the most important characteristics measured to define the population of nanoparticles, being the one mainly responsible for their special properties and also a main factor determining their toxicity. The most established technique for measuring the particle size distribution of nanomaterials is DLS. DLS requires the dispersion of particles in liquid, where they present random (Brownian) movement. The movement of particles is monitored by irradiating them with a laser and the temporal fluctuation of scattered radiation is transformed into an estimation of their hydrodynamic diameter (which includes the particle and solvation sphere and constitutes an estimation of the particle size). This technique assumes that the particles are spherical as described by Stokes-Einstein equation and in cases where these are not, there will be major discrepancy between the estimated size by DLS and microscopy (L. Mbundi et al. 2014). The theory of different modalities of light scattering have been the objective of a review (Brar and Verma 2011).

The determination of the particle size distribution will probably require purification of the nanosystems because the analysis can be greatly affected by surrounding particles or macromolecules. The bigger components of the sample need to be separated because they would lead to multiple scattering, rather than the required single scattering, and reduce interparticle interactions, which would also lead to inaccurate measurement: this can be done by filtering or centrifuging the samples. The purification required should not alter those factors that affect the dynamic circumference of the nanosystems: their diameter, shape, charge and electrical mobility of the particles. Temperature, pH, ionic strength and viscosity of the media

may affect the dynamic sphere. Therefore, preserving factors in the matrix affecting its dynamic circumference is a priority in this analysis.

Dilution of the particles will also reduce multiple scattering. The dilution can induce change in the morphology of organic nanosystems. For instance, some polymers, when diluted in aqueous media, can change towards orientating the most hydrophilic groups towards the exterior of the particle; and hydrophobic groups would orientate towards the inner part of the nanosystem or evolve towards forming agglomerates. If the dilution is carried out with the same solvent than in the samples, the organic nanosystems will not rupture or change shape. An example of critical dilution step carried out was the determination of the size distribution of nanoemulsions containing ß-carotene when were subject to conditions in *in vitro* simulated gastro-intestinal tract. The sample containing nanosystems were diluted 10 times with saliva fluid, gastric fluid and buffer at pH 7, to mimic the intestinal phase, prior to the analysis with DLS (Gasa-Falcon et al. 2017). In some cases, surfactant, such as the non-ionic Tween 20, assisted in dispersing nanodroplets and preventing their coalescence. However, substances such as bile salts, phospholipid and lipase, which are present in the intestinal phase, can displace Tween 20 and lead to increased droplet sizes, as found in investigations studying changes in nanoemulsions during the digestion (Gasa-Falcon et al. 2017). Besides the addition of a surfactant, the application of ultrasounds before measurements with DLS can reduce agglomeration, but the dispersion achieved will decrease with time; and it could also de-agglomerate nanosystems as they were in food, which would be undesirable.

The measurement of the Z-potential of the system (potential difference between the media and the stationary layer of fluid associated to the particle) with Laser Doppler Microelectrophoresis, can be carried out with the same instrument than DLS and will show if the nanosystems are stable under the conditions of the measurement or if, on the contrary, there are inter-particle interactions leading to agglomeration. Z-potential is typically measured when formulating and

measuring the stability of emulsions (X.-F. Zhu et al. 2018). Z-potential can change with the adsorption of biomolecules, and through this parameter, the effect of the different gastrointestinal phases on the physic-chemical properties of nanoemulsions can be monitored. For instance, a study found changes in the Z-potential along the gastro intestinal tract, especially in the stomach phase, which led to a reduction of the negative charge possibly because salts present could shield electrostatic interactions. On the contrary, the intestinal phase led to more negative Z-potential, reaching values as high as the initial state or in the mouth phase, possibly because the adsorption of bile salts or phospholipids from intestinal fluids (Gasa-Falcon et al. 2017). This example illustrates how the composition of the solvent can affect the properties measured in nanoemulsions.

The pH of the media used for the DLS and Z-potential measurements can also have an impact in the shape of the nanoparticle in solution, as what was studied in the particular case of the nanomaterial graphene oxide (Whitby et al. 2011). The effect of the pH will be more prominent in nanosystems with ionisable functional groups. Therefore, the dilution of the sample prior measurements with DLS can be necessary for the estimation of the particle size distribution, however it can also induce changes in the shape and size of the hydrodynamic sphere of the nanosystem. Currently, the smallest hydrodynamic spheres that can be detected are in the range of 0.3 nm (Marlvern Instruments Limited 2015), which is actually an overestimation of the particle size given that the hydrodynamic sphere includes solvent.

Microscopy imaging is considered a standard technique in the characterisation of nanomaterials (EFSA Scientific Committee 2011). It allows studying the direct interaction of the nanosystems with the matrix and provides information of their size and shape. Transmission Electron Microscopy (TEM) and SEM are the techniques most widely used in recent investigations. SEM micrographs originate from low energy secondary electrons scattered off from the sample after being scanned with a low energy beam of electrons (1-30 keV). SEM provides great depth

of field (images in 3D). In TEM, a high energy electron beam (80-300 keV) is transmitted through a very thin sample providing images with high resolution (Dudkiewicz et al. 2011; Busquets 2017). The resolution achieved with state-of-the art TEM is below 0.1 nm, and it is favoured by thinner samples and electron beam with high accelerating voltage. For soft samples such as food, which can become damaged during imaging, accelerating voltages of up to 100 KeV are recommended (Dudkiewicz et al. 2011).

The sample can be bulky and prepared without difficulties for SEM analysis. In contrast, thin sample are required in TEM and these are more challenging to prepare. Both techniques work under high vacuum and require dry samples. Other modalities of microscopy are more suitable than SEM and TEM for imaging in moist environment (environmental (E-), liquid-, wet- SEM/ TEM), or image under cryogenic conditions, which requires high vacuum and frozen specimens. Cryo-sectioning is useful for imaging semi-liquid samples or samples that cannot be fixed due to their composition; for scanning the internal structure of nanosystems with SEM; or when preserving the sample matrix is a priority (Dudkiewicz et al. 2011). E-SEM does not need coated samples, and can be used to imaging food in their natural state, and although it can achieve resolution below 1 nm, it offers less resolution than standard SEM. The wet- modality requires the samples to be encapsulated and it is especially useful for imaging nanoparticles of metals in liquid food samples in their native state (Lubinda Mbundi et al. 2014). These capsules can be centrifuged and coated which would allow to enrich a membrane with nanosystems (Dudkiewicz et al. 2011). However, these microscopy techniques that make possible imaging hydrated samples such as food do not have widespread use yet maybe because of the highly specialised equipment needed.

The preparation of the specimens with nanosystems for imaging can consist of relatively mild treatments such as: fixating the protein structure with glutaraldehyde; treating the lipid structure with osmium tetroxide; dehydrating with ethanol; and cryo-fracturing (Dudkiewicz

et al. 2011); embedding in resin; leaving the specimen to dry on air; freeze-drying; absorbing liquid with filter paper in contact with the sample drop to avoid agglomeration (Novak et al. 2001); or dispersing the nanosystems with surfactant (i.e., 0.1 % sodium dodecyl sulphate) before drying (Mokhtari, Jafari, and Assadpour 2017). Liquid food samples can be encapsulated in agar prior to the standard pre-treatments (Dudkiewicz et al. 2011). Specimens containing metallic nanoparticles, or specimens that can be treated with heavy-metal stain, can be imaged through high energy back scattered electrons in SEM to improve the contrast between elements with different atomic number within a complex matrix. This strategy offers clearer interpretation of the data; however, it is then recommendable to compare the image with a stained control sample.

To minimise charging effects and improve contrast in SEM, specimens can be coated with a conductive layer, typically from metal or carbon (i.e., non-conductive starch films were coated with a thin layer (<50 nm) of gold) (Dudkiewicz et al. 2015; López-Córdoba et al. 2017). A main electron source in SEM is Field Emission (FESEM). FESEM is commonly used to obtain high quality micrographs from soft substrates, such as rosemary nanoparticles or echium oil by scanning with low voltage (Comunian et al. 2017; López-Córdoba et al. 2017). FESEM results in low electrical charging and does not make necessary sputtering the samples with conductive coating. The sample preparation steps listed in this section involve mild treatments that preserve with certain extent the environment of the micro- and nanosystems within the food matrix, and are appropriate for imaging samples that contain relatively high concentration of nanomaterials. However, the usual case when studying nanoparticles that have migrated to food is having samples with low abundance of nanomaterials and this will translate into greater difficulty when having to locate them with the microscope, carrying a greater statistical uncertainty to the measurement.

Indeed, microscopic techniques have the limitation that they require a very small sample (i.e., a droplet of ~10µl) which can lead to inadequate statistical representativity of the bulk sample (Dudkiewicz et al. 2011). Furthermore, imaging can also be carried out in a very localised area of the specimen, and if the concentration of particles in the sample is low, detecting enough particles may become too challenging. If the number of particles was not enough for their robust measurement, preconcentrating should be considered. Hence, the development of protocols to recover enough particles for imaging from liquid and solid food samples are very important (Lari and Dudkiewicz 2014). Following examples of such strategies are illustrated through procedures to recover and pre-concentrate synthetic amorphous silica from tomato soup and spherical silver nanoparticles from meat while trying to preserve their clustering state in the samples. These procedures were selected by the authors after having tested others such as drying or ultracentrifuging. Soup samples were diluted with borate buffer at pH 8, conditions that led to a negative charged sample that is beneficial for electron microscopy analysis. A drop of the pre-treated soup sample was placed onto a TEM grid coated in 0.1 % solution of skin porcine gelatine. For SEM analysis, the samples were attached with carbon glue to the stub and coated with Pt/Pd. Frozen meat was diluted with the same borate buffer and homogenised. The homogenised samples spiked with particles were centrifuged in tubes which contained hydrophobic TEM grids supported onto agar supports. These protocols were found advantageous with respect to resin embedding and cryo-sectioning in terms of preparation time, less need of specialised equipment and increasing sample volume which increases the representativity of the sample (Dudkiewicz et al. 2015).

An excellent work assessing the preparation of samples for electron microscopy identified that the number of particles analysed was not a main contributor in the uncertainty associated with the measurement of their size. The number of particles that needs to be analysed to achieve uncertainty below 5 % ranged from 38 to 359 for particle sizes ranging from 34 to11 nm,

respectively (Dudkiewicz et al. 2015). In contrast, recent works tend to assess particle size distribution with lower number nanosystems (Comunian et al. 2017; Rasti, Erfanian, and Selamat 2017); guidelines informing about harmonised procedures to characterise particles with different techniques would assist the diverse community of scientists working in the field of food nanotechnology. In contrast, the homogeneity of the initial sample was found to be a main contribution in the uncertainty associated with the determination of the particle size distribution (Dudkiewicz et al. 2015). Hence, digesting the sample matrix or extracting the particles for greater homogeneity would be advantageous although it would reduce the meaning of the information. Imaging from greater number of independent sample replicates would effectively reduce uncertainty. Importantly, the food matrix could affect the reproducibility of the measurement of the particle size in SEM and TEM; the measurement was affected significantly just in one of the studied matrices (Dudkiewicz et al. 2015), which is in agreement with a previous study measuring Ag nanoparticles in meat (Grombe et al. 2015). Interestingly, the reproducibility obtained for the analysis of Ag nanoparticles when embedded in meat (RSD 3 %) was 3 time better than in stock solution. This could be because the meat matrix would have minimised particle clustering (Dudkiewicz et al. 2015). An evaluation of food sample preparation methods suitable for electron microscopy, as well as the establishment of a selection tree to aid in the selection of imaging methods have been published by Dudkiewicz et al. (Dudkiewicz et al. 2011). Careful considerations must be taken with the sample treatment strategy because it can alter the structure of the food matrix and agglomeration of the particles. It is recommendable to image the matrix following a range of sample preparation steps to realise the implications of these pre-treatments in the information obtained.

### 14.4 Separation and detection of nanosystems

Separation techniques have been used when quantifying the loading capacity of nanosystems. In this context, the amount of active principle encapsulated is analysed with methods developed for the determination of the molecules after breaking the nanocapsules. For instance, the concentration of polyphenols (synaptic acid and quercetin) and echium oil (rich in ϑ-3 fatty acids) in microcapsules was quantified by rupturing the capsules and extracting the active principles with liquid-liquid extraction using methanol. Polyphenols where quantified in the alcohol extract with UV-Vis using two different wavelengths, whereas the fatty acid were isolated using an extraction with hexane and evaporation to dryness (Comunian et al. 2017). In cases where the analytical test was highly selective regarding a property of the nanosystems, a simple separation has been carried out. For instance, the release of rosemary nanoparticles from Cassava starch film was assessed by shaking the film in food simulant and analysing the release nanoparticles in solution with Folin Ciocalteu UV-Vis assay selective to polyphenols, without needing to separate other molecules from solution (López-Córdoba et al. 2017).

The separation of a mixture of compounds integrating micro- and nanosystems or the separation of markedly different micro- and nanosystems from the same food matrix is scarce in the literature but it holds the key to solve complex problems that simple liquid-liquid extraction and UV-Vis analysis cannot solve. The quantification of molecular components of organic nanosystems can be carried out by rupturing the nanosystems and separating them with traditional liquid chromatography, gas chromatography (if the components are volatile or can be derivatised to volatile substances) and by capillary electrophoresis. A modality of capillary electrophoresis (micellar capillary electrophoresis) would allow the analysis of micelles directly. The development and validation of the analytical methods by these traditional separation techniques is neither challenging nor time consuming. The most suitable detection systems for these separation techniques are UV/fluorescence/mass spectrometry (for liquid chromatography and electrophoresis) and mass spectrometry (for gas chromatography).

There is the need to separate nanosystems based on their size, shape and differentiate between their agglomerate estates. These techniques should be high-throughput, ideally. The separation of the nanosystems can be done with size exclusion chromatography (SEC), which working range is 0.5-10 nm and hydrodynamic chromatography (10nm-2µm) (Peters et al. 2011). In SEC, the separation of particles is based on the nanosystems' hydrodynamic volumes, and not on the interaction of these with the stationary phase like in other chromatographic modalities. The separation in SEC is carried out using a packed beads column with porous beads. The separation of nanosystems based on their shape is possible with SEC, for instance, rod and spherical gold nanoparticles could be separated thanks to the addition of mixtures of surfactants in the mobile phase (Wei, G-T; Liu, F-K; Wang 1999). To minimise sorption of the nanosystems onto the stationary phase, surfactants such as sodium dodecyl sulphate may be added to the mobile phase and the separation can notably be improved (Wei and Liu 1999). SEC has been used for the separation of inorganic nanomaterials (Kowalczyk, Lagzi, and Grzybowski 2011) but it is not commonly used for the analysis of organic nanosystems purified from food yet (Busquets 2017). In hydrodynamic chromatography, the stationary phase is constituted by non-porous packed beads and therefore the matrix will not affect the separation, which makes it advantageous for the analysis of purified extract for food. Typical detection systems in hydrodynamic chromatography are UV, DLS and MS (Philippe and Schaumann 2014). Therefore, it can be used for both organic and inorganic nanosystems although, like SEC, its use is still rare in the analysis of organic nanosystems but it may progress thanks to the advantages it offers for the analysis of complex food matrices. Hydrodynamic chromatography (HDC) has proven to be useful when separating mixtures of nanoparticles (i.e ZnO, $TiO_2$) with high presence of organic matter and salts. It can separate by size and quantify nanoparticles with sensitivity in the part per billion level when coupled to mass spectrometry. The separation of polystyrene nanoparticles with HDC showed that it is possible to keep

agglomerates during the separation, even when the interaction between particles is weak, possibility that makes it very promising for the study of nanofood (Philippe and Schaumann 2014). Field Flow Fractionation (FFF) can separate macromolecules, microorganism or particles (1 nm-1 μm) based on their different mobilities. In this case the particles advance through a channel where there is aqueous mobile phase pumped through two tightly packed polymeric layers and the action of a field perpendicular to the hydrodynamic flow. The perpendicular field can be gravity (flow FFF), a centrifugation force (sedimentation FFF), which can have a greater resolution capacity than FFF, among other modalities (Fedotov et al. 2011). The detection of particles following FFF can be carried out with UV, fluorescence, DLS or MS, consequently it can be used for both inorganic and organic particles. FFF is a very mature and robust technique has great potential for the separation of nanosystems; the channel is relatively simple to operate, and even to make however, it requires method development and it is not widely available in the laboratories dealing with food technology. This may be holding back establishing this technique as a reference one for the separation of nanosystems in food.

### 14.5 Complementary analytical techniques

Every analytical technique can be applied within a defined working range of conditions or concentrations. Microscopy techniques are very important in the nanofood context because they can be used to measure direct properties in nanomaterials, unlike many other approaches that offer indirect information. Microscopy has drawbacks, such as the localised analysis and small specimens which can lead to problems of representativity of the whole sample, or the very high number of micrographs that need to be treated for establishing the particle size and its uncertainty. The dependence of the relative standard deviation of the median particle size

(RSDpm), where particle size had been estimated from the equivalent circular diameter of the particle as projected in a 2D image; with the number of particles (N) and interquartile range of particle size distributions (IQR %) is given in the equation 1 (Dudkiewicz et al. 2015).

$$RSDpm = 10071xN^{-0.553}xIQR\ \% \qquad \text{(Eq. 1)}$$

Equation (1) was obtained from the measurement of a population of 1388 particles randomly selected from 200 images from the analysis of reference food materials (chicken paste and soup) spiked with silver and silica nanoparticles. The smallest number of particles required for an IQR(%) of particle size 111 nm, with an RSDpm of 5% was found to be 359 particles (Dudkiewicz et al. 2015).

The sample preparation method may be selected after trying several approaches for a nanosystem/food matrix to understand how preparation can affect the details in the image. It is highly recommendable to characterise nanosystems with a range of techniques for both sample treatment and determination.

An example of the advantages of the analysis with complementary microscopy techniques is the optimisation of a nanoemulsion-filled hydrogel, developed to improve the bioavailability of nobiletin, which is a flavone with pharmaceutical properties (Lei et al. 2017). This example is shown in Figure 1. A hydrogel filled with nanoemulsion containing nobiletin was freeze-dried and its morphology examined with SEM. The loading of the active principle of the hydrogel was found to affect the morphology (undulation) of the hydrogel with SEM. A detailed morphological examination of the crystals of the active principle dispersed in the hydrogel was carried out with optical and fluorescent microscopy. With optical microscopy,

the crystals presented filament structures and were especially visible at the higher loading concentrations of the drug (indicated with arrows in Figure 1). A convenient pre-treatment was carried out to observe the nanoemulsion within the hydrogel: a fluorescent hydrophobic dye (nile red) was dispersed in the nanoemulsion before drying the hydrogel. This pre-treatment led to a very clear picture of the distribution of the dye, which could interact with the hydrophobic active principle within the hydrogel. The crystals of the hydrophobic drug seemed to favour coalescence of hydrophobic droplets around them. In addition, the analysis of the hydrogel with XRD indicated that the active principle was mainly in amorphous form within the hydrogel and in less extent as crystals (Lei et al. 2017). This had implications in control release of the drug. An alternative technique that could analyse crystalline samples, through interference contrast, is High Resolution TEM (HRTEM) (Dudkiewicz et al. 2011).

The analysis of alginate nano/microspheres loaded with peppermint phenolic extract carried out with SEM, TEM and DLS (Mokhtari, Jafari, and Assadpour 2017) showed the advantages of a multianalytical approach to characterise nanocapsules (shown in Figure 2). In SEM, the alginate hydrogel is shown as a microparticle (>1 μm) that may be constituted from agglomerated sub-particles. The agglomerate may be an artifact which may have formed when drying the sample on air. We assume that that micrograph is representative of the sample, but certainly, additional images from the nanocapsules would be informative. The micrograph was obtained with rather high accelerating voltage (15 kV) given that alginate is a soft material and the specimen could suffer modifications by the electron beam during imaging: the selected voltage has to be high enough to achieve optimal resolution but low enough to not to alter the sample and prevent charging. In contrast to the result obtained with SEM, the TEM micrograph shows a 2D image of a population of nanocarriers with a range of particle sizes, after having treated the sample with surfactant. The surfactant stabilised the emulsion droplets and minimised coalescence, but may have de-agglomerated existing clusters in the original sample.

The particle size distribution shown by dark-field TEM (detection of the fraction of the beam diffracted by the sample) agrees with the size distribution of the hydrodynamic sphere obtained with DLS. The measurement with DLS was probably carried out after a filtration step to eliminate bigger particles.

CLSM is an advantageous scanning probe microscopy technique. It can achieve poorer spatial resolutions (50-100 nm) and lower magnifications than SEM or TEM but it can be used to image nanosystems in food samples that have been de-hydrated and fixed onto a slide or even hydrated samples. It is possible to obtain images in 3D and in colour when different parts of the sample have been died with auto fluorescent dyes (Prasad, Semwogerere, and Weeks 2007; Lubinda Mbundi et al. 2014). CLSM can be very useful to image nanosystems within food matrix (Salvia-Trujillo, Decker, and McClements 2016), and also to study the fate of nanosystems in cells and tissues when investigating their safety.

A complementary technique to DLS and microscopy that allows establishing the particle size distribution is Nanoparticle Tracking Analysis (NTA), which started to be commercialised (in 2006. This technique combines laser light scattering microscopy with a charge-coupled device camera that records the trajectory of nanoparticles which have Brownian movement when suspended in solution. The movement of the particle can be related with their size with a formula derived from the Stokes-Einstein equation. The particle size range of NTA (30 nm-1µm) is slightly shorter than electron microscopy and DLS (1nm-1µm), and it cannot detect sub-nanometer particles like TEM (Filipe, Hawe, and Jiskoot 2010). The concentration range where NTA operates ($10^7$–$10^9$ particles/ml) is narrower than the range for DLS ($10^8$–$10^{12}$ particles/ml) (Filipe, Hawe, and Jiskoot 2010). NTA is particularly useful for characterising monodisperse and polydisperse samples and can have superior peak resolution than DLS. Different populations of particles can become very well defined with NTA and the presence of bigger particles does not affect the detection of the smaller ones, unlike in DLS; this is an

important advantage. NTA also allows studying aggregation at different temperatures with the instrument and provides information about the aggregation kinetics. A limitation is that the analysis time with NTA (5-60 min) can be longer than with DLS (2-5 min) (Filipe, Hawe, and Jiskoot 2010). But overall, it is a very powerful characterisation technique, which is not widely used in the characterisation of nanosystems in food and their aggregation behaviour, possibly because it is relatively new in the market and DLS is, in contrast, a very well-established technique. The current definition of nanomaterial indicates that 50 % of the particles, in either free, aggregates or agglomerates form, possess structures in the critical size range (below 100 nm) (Potocnik 2011). Consequently, the capacity to measure size and size-range of the particles needs to be prioritised when deciding on the method and technique to characterise nanotechnology based materials, and both NTA and DLS offer this possibility, whereas it would be an enormous task by microscopy as vast number of micrographs would need to be treated.

A study compared the uncertainty of the measurement of particle size of pristine silica particles with SEM, DLS and GEMMA (Gas Electrophoretic Mobility Molecular Analyser). In GEMMA, single charged analytes are produced by the action of an electrospray and charge reduction with polonium-210. Following, charged nanoparticles are separated by their electrophoretic mobility (Allmaier, Laschober, and Szymanski 2008). These three techniques showed an uncertainty of 3-6 % in the measurement. In contrast, when comparing DLS, GEMMA and TEM for the analysis of Ag nanoparticles in aqueous dispersion, the uncertainty obtained with TEM (8 - 21 %) was like with DLS and about 2 times greater than with GEMMA. The cause of the relatively high dispersion of results could be sample inhomogeneity, sample preparation or data treatment. TEM was selected instead of SEM in this case because it gave greater contrast between the nanomaterials, which were imaged as dark spots, and the matrix, in bright-field TEM.

Quantitative analysis of silica nanoparticles in soup and Ag nanoparticles in meat were carried out with FFF-ICP-MS and Single Particle-ICP-MS (SP-ICP-MS), respectively, following matrix digestion. The analysis of Ag nanoparticles with SP-ICP-MS lead to up to 5 % uncertainty (compared to up to 19 % with TEM). The lower uncertainty achieved with the hyphenated techniques compared to microscopy was attributed to the higher homogeneity of the sample due to the digestion being carried out. The analysis of silica nanoparticles with FFF-ICP-MS led to up to 21 % uncertainty, which was similar to SEM. This high dispersion of the results could be due to intrinsic inhomogeneity of the sample (Dudkiewicz et al. 2015). Overall, hyphenated techniques can be advantageous with respect to microscopy: they reduce the effect of the matrix, and increase representativity of the sample, although method development with hyphenated techniques is time consuming. A scheme displaying the capabilities of a range of techniques discussed in this chapter is shown in Figure 3. SEM-EDS and XRD have the capacity to offer structural and compositional information (metals). These, and UV-Vis, could also be employed for the quantification of particle sizes, although this is normally carried out by DLS, which is not restricted by the composition of the particles and does not need high number of acquisitions (as in microscopy) or crystallinity (like XRD). Likewise, CLSM, ESEM, and HRTEM are mainly, but infrequently, used for providing qualitative information of the structure of nanosystems, and are seldomly employed for the measurement of particle size distribution. It is predicted that with the development and validation of analytical methods involving separation of particles using FFF, SEC and HDC, the analysis with hyphenated techniques will become more generalised.

## 14.6 Conclusions

Particle size, shape and the stability, distribution and evolution of nanosystems as well as the active principles that they may contain, within food products need to be established. Every analytical technique offers partial information of the situation in the nanofood or food packaging, and the limits of the information that every technique can offer with reliability are being defined. It is highly recommendable to characterise nanosystems with a range of techniques for both sample treatment and determination. SEM and TEM are widely used because they can show the shape and detail of the nanosystems, although advances that both techniques can incorporate for imaging hydrated samples are not fully exploited. Confocal microscopy can also be employed to characterise nanosystems in moist samples despite that it has limitations regarding the magnification that it provides. Major disadvantages of microscopy in quantitative analysis are the high number of samples that need to be imaged and treated to determine particle size and minimise problems of sample representativity that result from the small sample volumes required. The sample preparation method needs to be optimised for every study nanosystem/matrix as it is crucial to obtain accurate information in microscopy, but also in every analytical technique analysing food. DLS and NTA are excellent approaches to measure size distribution of nanoparticles. NTA is more robust than DLS in the sense that NTA's measurements are less affected by the presence of small amounts of large particles. Methods including hyphenated techniques can minimise matrix effects and separate particles by their shape and size and quantify nanosystems with high sensitivity (comparable to the analysis of organic molecules and metals). However more efforts are needed to develop methods that would facilitate the use of hyphenated techniques for the characterisation of nanosystems in food. Separation techniques such as HDC and sedimentation FFF coupled to mass spectrometry or DLS have a brilliant future ahead in the analysis of nanosystems in food.

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Figure 1 Microstructure of Nobiletin-loaded nanoemulsion-filled alginate hydrogels, I: SEM images of the hydrogel surface, II: images by optical microscopy, III: images by fluorescent microscopy. Arrows indicate the nobiletin crystals. The scale bar in I and II is unclear.

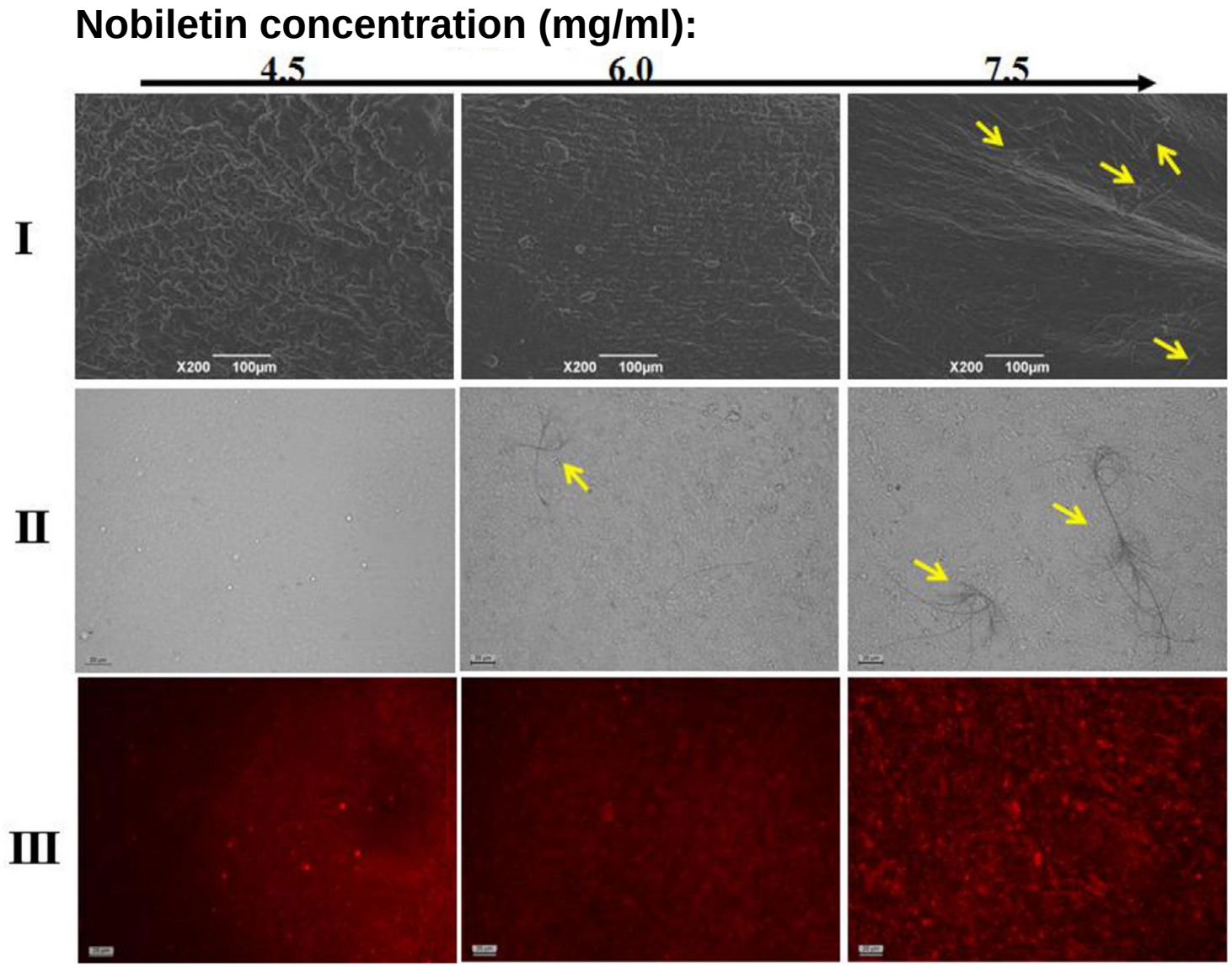


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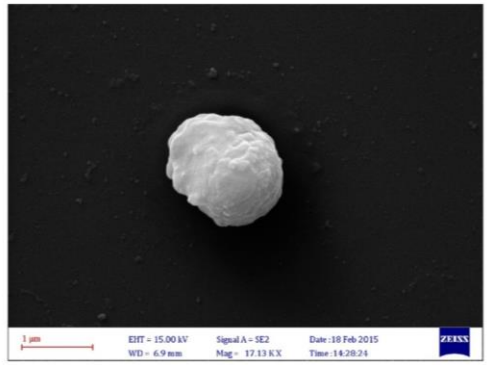
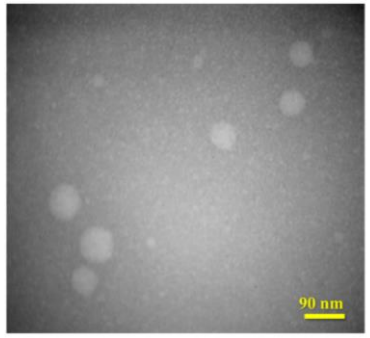
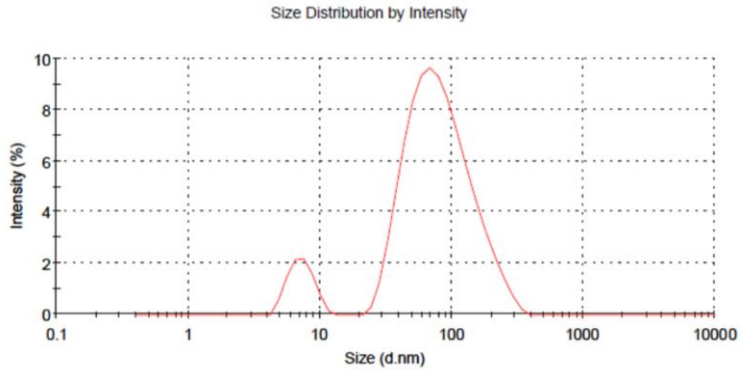
Figure 2 Alginate nanospheres aded with peppermint phenolic extract: (a) SEM photomicrograph, (b) TEM photomicrograph, (c) Size distribution graph. The scale bar in (a) corresponds to 1µm and in (b) corresponds to 90 nm.




a




b




c

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Figure 3 Classification of the scope of techniques used in the characterisation of organic or inorganic micro – and nanosystems in food. The most commonly used techniques within the scope of this chapter appear in bold.