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Rome, Istituto Superiore di Sanità
September 27, 2013

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This volume gathers the presentations delivered at the conference organized by the Department of Food Safety and Veterinary Public Health, the first national meeting devoted to nanomaterials in the food sector. The presentations, displayed in a concise form, are complemented by materials related to the conference and useful for the understanding of the themes and approaches dealt with by the various speakers. Overall, the volume provides an overview of the applications, regulation, analytical determination, risk assessment of nanomaterials in food products.
Key words: Nanomaterials; Nanoparticles; Food safety; Risk assessment

Istituto Superiore di Sanità
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Questo volume raccoglie le relazioni presentate durante il convegno organizzato dal Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare dell'Istituto Superiore di Sanità, il quale ha rappresentato il primo incontro nazionale dedicato ai nanomateriali nel settore alimentare. Le relazioni, proposte in forma sintetica, sono integrate con materiali inerenti il convegno stesso e utili alla comprensione delle tematiche e degli approcci trattati dai diversi relatori. Nell'insieme il volume offre una panoramica sulle applicazioni, la normativa, la determinazione analitica, la valutazione del rischio dei nanomateriali nei prodotti alimentari.
Key words: Nanomateriali; Nanoparticelle; Sicurezza alimentare; Valutazione del rischio

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PRESENTATION

Nanosciences and nanotechnologies are a broad interdisciplinary area of research, development and industrial activity that involves the manufacture, processing and application of materials having one or more dimensions of the order of 100 nanometres (nm) or less. Rapid advancements in nanosciences and nanotechnologies in recent years have opened up new prospects and have been predicted to offer several benefits with a significant impact on almost all industrial sectors, with the food sector in the forefront. At the same time concerns have been raised on potential risks related to the interactions of nano-sized materials at the molecular or physiological levels, which may ultimately harm human health and the environment. Since nano-sized materials can have quite different biological (and toxicological) activities as compared to the corresponding non nano-sized materials, new concepts and tools for safety assessment of nanomaterials are needed.

The assessment of the potential risks arising from nanoscience and nanotechnologies on food safety is complex and requires innovative approaches. Since several years now the Department of Food Safety and Veterinary Public Health of the Istituto Superiore di Sanità (the National Health Institute in Italy) is committed to the assessment of the potential impact of the use of engineered nanomaterials on food safety and consumers' health. The participation in the activities aimed at the development of analytical methods for the determination of nanomaterials in food promoted by the European Commission, the assessment of potential risks within the EFSA (European Food Safety Authority) Scientific Network for Risk Assessment of Nanotechnologies in Food and Feed ("NanoNetwork"), the contribution to European nanotoxicological projects recently concluded (NANOGENOTOX) or ongoing (NANoREG) are part of such commitment.

This volume gathers the presentations delivered at the conference "Nanomaterials in the Food Sector: New Approaches for Safety Assessment", organized by the Department of Food Safety and Veterinary Public Health on 27 September 2013. The presentations are complemented by materials related to the conference, to support a comprehensive understanding of the themes and approaches dealt with by the various speakers. In this first national conference devoted to nanomaterials in the food sector, the experts of the Department, along with selected experts from the European Commission (DG Joint Research Centre, Ispra), the European Food Safety Authority (EFSA) and research centres offered an up-to-date overview of this rapidly growing area from the standpoint of food safety. Technical and scientific references were provided to facilitate the comprehension of the updates and developments in the safety assessment of engineered nanomaterials in food. The meeting was held under the patronage of the Ministry of Health, which also contributed by providing a picture of the legislative framework in the area of novel foods and food labelling. The latter issue is of topical relevance, since Regulation (EU) No. 1169/2011 on the provision of food information to consumers prescribes the labelling of nanomaterials in food as of December 2014.

On the whole, the meeting was a major starting point to stimulate interest from researchers, stakeholders and the public and to promote discussion on cutting-edge topics for science and risk assessment in the agri-food system. Therefore, the present volume provides an overview of the issues relevant to applications, regulation, analytical determination, risk assessment of nanomaterials in food products along with some reflections and perspectives.

Francesco Cubadda
Conference chairman
National scientific expert in the EFSA Network
for Risk Assessment of Nanotechnologies in Food and Feed

PART 1
Contributions to the conference

APPLICATIONS AND PROSPECTS OF NANOTECHNOLOGIES IN THE FOOD SECTOR

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The exciting properties exhibited at the nanoscale have led to the development of vast numbers of different types of Engineered Nanomaterials (ENMs) also for the food industry.

Food packaging is a key area, with nanotechnology-derived polymers offering new solutions to keep food secure during transportation, increase the shelf life of products and protect them from pathogens (1). Other nanotechnology applications in the food sector range from altering the texture of food, to encapsulating food components or additives, to increasing the bioavailability of nutritional components, and to developing nano-sensors for traceability and monitoring the condition of food during the value chain (1). Whilst such developments potentially offer enormous benefits, they have also raised a number of issues in relation to consumer safety and environmental impacts, involving ethical, policy and regulatory issues. Indeed, the unusual properties of nanomaterials make it difficult to predict their biological reactivity; therefore, it is essential to be able to assess the risk associated with their use. This is why food nanotoxicology is an important and emergent field.

A number of nano-sized additives and supplements for food and healthfood products, and nanotechnology derived food packaging materials, are already available in some countries, and their number is expected to increase in the coming years. In terms of market volume the main categories on the global market (i.e., not limited to food applications) include inorganic non-metallic ENMs (e.g., synthetic amorphous silica, aluminium oxide, titanium dioxide), carbon based ENMs (e.g., carbon black, carbon nanotubes), metal nanoparticles (e.g., nanosilver) and organic, macromolecular or polymeric particulate materials (e.g., dendrimers) (Table 1) (2).

Table 1. Global market volume (i.e. not limited to food applications) of main engineered nanomaterials

Material	Volume (t)
Carbon black	9.6 million
Synthethic amorphous silica	1.5 million
Aluminium oxide	200 000
Barium titanate	15 000
Titanium dioxide	10 000
Cerium oxide	10 000
Zinc oxide	8 000
Carbon nanotubes and nanofibres	*several hundreds/few thousands*
Nanosilver	*ca. 20*

In terms of industrial impact and public exposure the above-mentioned ENMs are of immediate regulatory relevance (2). In particular, carbon black and amorphous silica represent by far the largest volume of ENMs currently on the market. Together with a few other ENMs, they have been on the market for decades and are used in a wide variety of applications. The group of materials currently attracting most attention comprises nano-titanium dioxide,

nanozinc oxide, fullerenes, carbon nanotubes and nanosilver. Those materials are marketed in clearly smaller quantities than the traditional ENMs, but the use of some of these materials is increasing fast (2). So far, in the EU three ENMs are authorized as additives for plastic materials and articles intended to come into contact with food, *i.e.* carbon black, silicon dioxide and titanium nitride (3).

Since several years, the team dealing with food nanomaterials in the Department of Food Safety and Veterinary Public Health of the ISS has been carrying out studies in the following three main areas (4-8):

- analytical determination of ENMs in food and biological tissues;
- *in vitro* studies to assess ENM modification/dissolution/degradation after ingestion (simulated gastrointestinal digestion models);
- *in vivo* oral toxicity studies (ADME/biodistribution, repeated dose toxicity, effects on endocrine/development/reproductive system, etc.).

The objective of these activities is, on the one hand, the development of analytical methods for the determination of inorganic ENMs in food and, on the other hand, the generation of new data to support risk assessment of food ENMs by the National Authority and the EFSA.

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ENGINEERED NANOMATERIALS: AUTHORIZATION UNDER NOVEL FOOD REGULATION (EC) NO 258/97

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In order to guarantee the public safety, the market of foods that have not been consumed to a significant degree in the European Union earlier has to undergo a premarketing authorization procedure to assess the safety for human consumption according to Regulation (EC) No 258/97 (hereafter named 'the regulation') (1).

According to the regulation, novel food and novel food ingredient that do not have a significant history of consumption before May 1997 can be divided into the following categories:

i) foods and food ingredients with a new or intentionally modified primary molecular structure (letter c in the regulation);

ii) foods and food ingredients consisting of or isolated from microorganisms, fungi or algae (letter d in the regulation);

iii) foods and food ingredients consisting of or isolated from plants and food ingredients isolated from animals, except for foods and food ingredients obtained by traditional propagating or breeding practices and having a history of safe food use (letter e in the regulation);

iv) foods and food ingredients to which has been applied a production process not currently used, where that process gives rise to significant changes in the composition or structure of the foods or food ingredients which affect their nutritional value, metabolism or level of undesirable substances (letter f in the regulation).

The Regulation does not cover food for which an approval procedure is already in place, such as food additives, flavourings, extraction solvents and GMOs (Genetically Modified Organisms). If food was used exclusively in food supplements, new uses in other food require authorisation under the novel food Regulation (e.g., food fortification requires authorisation).

To market a novel food, companies must apply to the competent authority of one of the member states for authorisation, presenting a dossier, containing all the scientific information to support the safety of the product. When the competent authority decides that no additional assessment is necessary and if the Commission and EU member states do not object the product can be marketed in the EU under an authorization issued by the member state. Otherwise, an additional assessment is necessary and the approval or refusal of marketing of the product is stated in a Commission decision, approved after receiving the opinion of the Standing Committee on Food Chain and Animal Health (Figure 1).

In some cases, a novel food included in the categories ii) and/or iii) (see list above) may be marketed through a simplified procedure called "notification". The company notifies the Commission about the marketing of a novel food or ingredient based on the opinion of a food assessment body of one of the member states that has established "substantial equivalence".

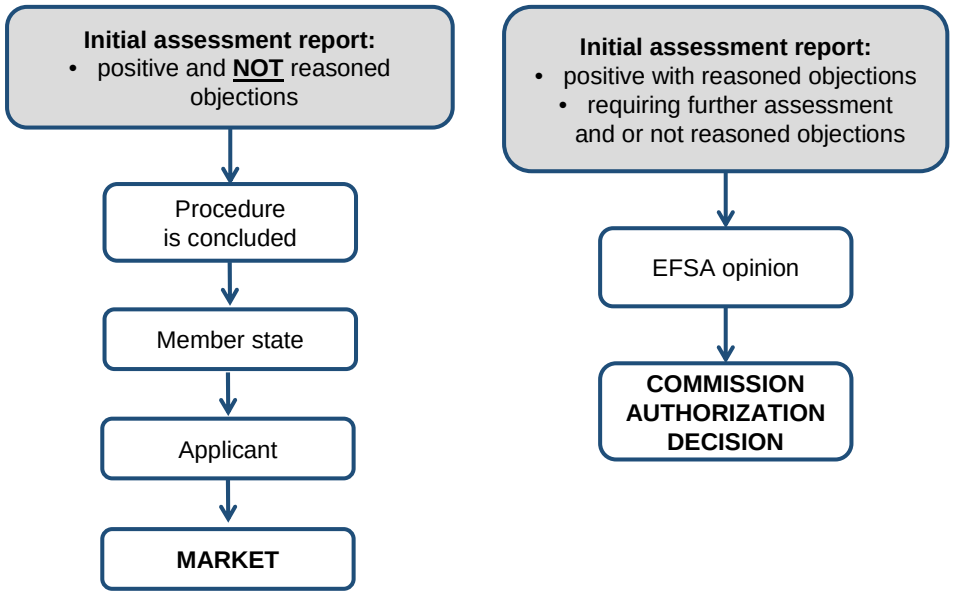

Figure 1. Main authorization procedure of a novel food under Regulation (EC) No 258/97

Foods different from additives, flavourings or enzymes that can be classified as engineered nanomaterials are considered novel in the meaning of category iv) of the above list. The authorization procedure must be followed prior to their marketing.

A proposal of a new regulation on novel food was adopted in 2008 but failed in 2011. A new proposal has been presented in December 2013 and the discussion at the Council are expected to begin in early 2014. As the former one, this new proposal explicitly makes reference to engineered nanomaterials.

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LABELLING OF NANOMATERIALS IN FOOD: THE REGULATION (EU) No 1169/2011

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On 25 October 2011, the European Parliament and the Council adopted the Regulation (EU) No 1169/2011 on the provision of Food Information to Consumers (FIC Regulation) (1).

The FIC Regulation modifies existing food labelling provisions in the European Union to allow consumers to make informed choices and to make safe use of food, while at the same time ensure the free movement of legally produced and marketed food. It entered into force on 12 December 2011. It shall apply from 13 December 2014, with the exception of the provisions concerning the nutrition declaration which shall apply from 13 December 2016.

In order to inform consumers of the presence of engineered nanomaterials in food, Article 18(3) of Regulation (EU) No 1169/2011 provides that all ingredients present in the form of engineered nanomaterials must be clearly indicated in the list of ingredients and the names of such ingredients must be followed by the word "nano" in brackets. In addition, it provides a definition of engineered nanomaterials, which may be adjusted and adapted to technical and scientific progress or to definitions agreed at international level, by means of delegated acts, for the purposes of achieving the objectives of the Regulation.

On 18 October 2011, the Commission adopted Recommendation 2011/696/EU on the definition of nanomaterial (2). According to the Commission Communication to the European Parliament, the Council and the European Economic and Social Committee on the Second Regulatory Review on Nanomaterials, the Commission intends to use the definition of 'nanomaterial' set out in the Commission Recommendation 2011/696/EU in EU legislation and instruments of implementation, where appropriate. Where other definitions are used in EU legislation, provisions will be adapted in order to ensure a consistent approach, although sector specific solutions may remain necessary.

On the basis of Article 18(5) of Regulation (EU) No 1169/2011, for the purposes of achieving the objectives of the Regulation, the Commission shall, by means of delegated acts, adjust and adapt the definition of engineered nanomaterials to technical and scientific progress or to definitions agreed at international level. The Commission thus decided to adapt the definition of 'engineered nanomaterials' laid down in Article 2(2)(t) of Regulation (EU) No 1169/2011 to the Commission Recommendation 2011/696/EU, taking into account the necessary sector specific considerations, by a Delegated Regulation.

The new definition has been discussed within the Working Group with experts from Member States set up by the Commission's Health and Consumer Directorate General and the draft delegated act has now been notified to the World Trade Organization (WTO) under the Technical to Barriers Agreement (TBT notification).

Table 1 reports the Timeline for the enforcement of Regulation (EU) No 1169/2011 on labelling of engineered nanomaterials in food.

Table 1. Timeline for the enforcement of Regulation (EU) No 1169/2011 on labelling of engineered nanomaterials in food

Event	Timing
Preparation of a draft delegated act (agenda Working Group)	Already done
Consultation with stakeholders	23 May 2013
Notification of draft delegated act to WTO	11 September 2013
Comments of WTO members to the EU	until 10 November 2013
Adoption of the delegated act by the Commission. Evaluation by the European Parliament and the Council, which may exercise their right of objection	to be defined
Right of scrutiny of EP/Council	2+2 months following adoption
Publication in the Official Journal (if no objections)	March 2014 at the latest
Food Business Operators have time to adapt the labelling	until 13 December 2014

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ANALYTICAL DETERMINATION OF NANOMATERIALS IN FOOD

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Analytical methods normally need to answer two questions: 1) whether a certain substance is in the sample (identity); 2) how much of the substance is in the sample (for a nanomaterial – hereafter NM – mass or number of particles). Both identity and quantity define the traceability of the analytical result.

Determination of nanoparticles presents analytical challenges for definition of identity as well as quantification (1). The term "identity" consists of two parts, namely size and chemical composition. For known single chemical substances, mass fractions (e.g., mg/kg) can easily be converted to amount of substance fractions (mol/kg) using the molar mass of the substance. No such simple conversion from mass fractions to number of particles exists for nanoparticles, as particles of the same chemical composition can differ in size, mass and density. As far as size is concerned, additional challenges are that:

a) every non-spherical particle can be characterised by multiple "sizes";
b) sizes may differ between the dried or dispersed state of a particle and measured sizes are always method-defined.

Different particle size analysis techniques measure fundamentally different parameters, nonetheless all output is called ''size''. Examples of the output of size measurements with different techniques are:

– *Transmission electron microscopy*
  lateral dimensions of a 2D projection of the particle, usually in dry state (average diameters are either average, median, or modal values obtained from the number-based size distribution).

– *Dynamic light scattering*
  hydrodynamic diameter of particles (expressed as diameter of a spherical particle that has the same Brownian motion behaviour in suspension).

– *Centrifugal liquid sedimentation*
  sedimentation behaviour of a particle and expresses size as diameter of a sphere of equal sedimentation properties.

As far as quantity is concerned, it has to be highlighted that unless the particles are near-spherical and of uniform and known density, mass based, number based, and intensity based results, which are by definition not comparable, may also be virtually non-convertible (1).

Based on the nanodefinition of the European Commission (2), validation of methods for detecting and quantifying nanoparticles in food must answer three questions:

i) Are there nanoparticles in the sample? (size identity);
ii) If yes, what kind of particles? (chemical identity) and
iii) How much NM is in the sample? (mass or number fraction).

Both size identity and quantity of nanoparticles are intrinsically method-defined. Direct comparison of results from different methods is therefore only possible if several assumptions

on the shape and density of the particles are made. This limits the concept of the screening/confirmatory method.

Overall, all the metrological and conceptual problems highlighted above can be solved by a clear definition of the measurand. However, there are also practical challenges related to detecting and quantifying nanoparticles in food. The particulate nature of the analyte may cause problems in the final quantification step, for insance in imaging by electron microscopy only a small fraction of the analytical portion is studied whereas it must be ensured that a sufficiently large number of particles are counted. Another problem is that particles may undergo changes during sample preparation and final quantification (such as agglomeration, disagglomeration, or even dissolution). Finally, the different sensitivity to different particles of the same kind should be kept in mind as a potential problem (properties of nanoparticles may vary from producer to producer and even from batch to batch due to differences in morphologies, stabilising agents, etc.).

The most important techniques for measuring nanoparticles size distribution are centrifugation-based techniques (e.g. Centrifugal Particle Sedimentation, CPS; and Analytical Ultracentrifugation, AUC), laser light-scattering techniques (e.g. Dynamic Light Scattering, DLS), electron microscopy (e.g. Scanning Electron Microscopy, SEM; and Transmission Electron Microscopy, TEM), hyphenated ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) based methods (e.g. hydrodynamic chromatography ICP-MS/HDC-ICP-MS and Field Flow Fractionation ICP-MS/FFF-ICP-MS), and single particle ICP-MS (3). However, only ICP-MS-based techniques are able to also determine the chemical identity and this, along with their speed allowing for a reasonable sample throughput, makes them the most promising for the detection and characterisation of inorganic NMs in food.

Irrespective of the analytical technique used, a proper measurement of nanoparticles requires a simplification of the matrix into which they are embedded, which call for extensive, carefully executed and documented sample preparation. In general, extracting the nanoparticles from the embedding matrix is required and it must be ensured that the sample preparation treatment does not modify the original particle size distribution.

So far, three interlaboratory studies have been carried out by the EC on the determination of Ag particles in food simulants/matrices, two of them by single particle-ICP-MS and one by AF4 (Asymmetric Flow Field Flow Fractionation)-ICP-MS. The team dealing with food NMs of this Department is one of the few laboratories worldwide that participated in all of these exercises. Besides nano-Ag, the team has focused on the determination of nano-SiO_2 and TiO_2 (4, 5). The determination of these NMs presents great challenges when an ICP-MS-based analytical platform is used. The way these challenges were addressed is discussed, with focus on chemical resolution of polyatomic interferences on Si and Ti masses by gas phase reactions in the quadrupole.

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DIETARY EXPOSURE TO NANOMATERIALS

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The principles of exposure assessment of Engineered Nanomaterials (ENMs) *via* food and feed are basically the same as in exposure assessment of non-nanoform materials (1). The starting point for determining the amount of ENM for the exposure assessment currently has to rely on information on the material added to food/feed or that is in contact with food/feed (1). The initial characteristics of the added ENM can be used as an assumption in the exposure assessment, but it is preferable to determine the amount of the ENM present in the food/feed matrix. In fact, the structure of the ENM in food/feed may be changed in the food/feed production chain during processing or storage because of their interactions with proteins, lipids and other substances present in the food/feed matrices. Hence, effects of processing and storage and the stability of the ENM should be accounted for. For ENM added to feed, the potential carry over to food should be considered for human exposure, which could be determined by measurement of the ENM in relevant animal tissue or products.

Use scenarios where it can be anticipated that consumer exposure does not arise include food contact materials with no nanomaterial migration and ENMs that are soluble or biodegradable, included delivery systems for bulk substances in nanoscale (for instance micelles, nanoemulsions or other encapsulation). On the other hand, there are cases where dietary exposure to nanomaterials is likely to occur and inorganic, insoluble and potentially bio-persistent nanoparticles are of particular concern in this respect. In general, the following are indicators of a potential for high exposure:

- high production volume for the field of application;
- high mobility of the nanoform in organisms, i.e. probability of internal exposure (e.g., transport via macrophages; transport through cell membranes, blood-brain barrier and/or placenta) and mobilization potential (e.g., infiltration, sorption, complex formation);
- targeted or controlled release;
- persistence/stability (e.g., in water, fat, and body fluids, lack of solubility/degradation);
- bioaccumulation.

For thorough exposure assessment, the determination of the amount and characterisation of the ENM present in the food as consumed, i.e. prepared as ready-to-eat (including cooking, etc.), is needed. However, it should be kept in mind that the ENM can undergo major alterations during the transit in the human gastrointestinal tract. Gastrointestinal digestion may degrade/dissolve the ENM turning it into the corresponding non-nanoform and such transformation may be complete or partial. On the other, nanoparticles with new properties may be formed and the amount of particles in the nano range can increase as compared to the original food. In general, substantial modifications are to be expected (e.g., in terms of agglomeration, surface charge), even due to the effects of degradation of the matrix on ENM characteristics, with unpredictable effects on absorption and potential toxicity. This highlights the importance of evaluating the effect of gastrointestinal digestion.

In vitro digestion methods can be used to assess the stability of the ENM in the gastrointestinal tract. With an *in vitro* digestion model, the conditions of the human

gastrointestinal tract can be simulated, i.e. temperature, mixing, transit time, composition of salt, enzymes and other constituents such as bile. When evidence is provided convincingly demonstrating, by appropriate analytical methods, that an ENM completely dissolves/degrades in the gastrointestinal tract, the hazard identification and hazard characterisation can rely on data for the non-nanoform substance as long as the possibility of ENM absorption before the dissolution/degradation stage can be excluded.

In the absence of such detailed exposure data and where it is not possible to determine the nanoform in the food/feed matrix, it should be assumed that all added ENM is present, ingested and absorbed in the nanoform.

Nano-SiO_2 and TiO_2 are appropriate examples of nanoparticles to which consumers are already exposed. In bulk form they are authorised food additives (E551, E171) but contain a nano-sized fraction (2, 3). Nano-SiO_2 and TiO_2 are produced in high tonnage volumes with present and/or prospective uses in the food sector and are potentially bio-persistent nanoparticles. A recent study addressed the effect of gastrointestinal digestion on nanosilica and found that upon consumption of foods containing E551, the gut epithelium is most likely exposed to nano-sized silica (4). Overall, it appears that further studies on this critical aspect are definitely needed.

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FOOD NANOTOXICOLOGY AND RISK ASSESSMENT

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The traditional risk assessment paradigm (hazard identification, hazard characterization, exposure assessment and risk characterisation) is considered appropriate for evaluating nanomaterials (NMs) from a food safety point of view (1). Similarly to other substances potentially present in food, NMs are a heterogeneous group with respect to their chemical, biological, physiological, pharmacological and toxicological behaviour. The risk of an ENM depends on its chemical composition, physico-chemical properties, its interactions with tissues, and potential exposure levels (1). The European Food Safety Authority (EFSA) has highlighted the need for generating new data and approaches able to cope with NM characteristics affecting their biological activity (higher reactivity and bioavailability as compared to the non-nanoform) and modulating their toxicological properties with mechanisms and effects still to be elucidated.

Among the NMs of greater relevance in the food sector titanium dioxide and silicon dioxide rank high. TiO_2 in bulk form is a food additive approved by the European Union (E171) and often present in sweets. A recent study has shown that in food grade TiO_2 ca. 36% of the particles may be <100 nm in at least one dimension, indicating potentially significant dietary exposure to nano-TiO_2 especially for children (2).

In this respect, the possible effects on reproductive, endocrine and immune system after repeated oral administration of nano-TiO_2 to rat at dose levels (0, 1, 2 mg/kg body weight per day) compatible with potential human intake have been studied (3). Nanoparticles were characterised by scanning electron microscopy and transmission electron microscopy, and their presence in spleen, a target organ for bioaccumulation, was investigated by single-particle inductively coupled plasma mass spectrometry and SEM/EDX. Results show that TiO_2 nanoparticles administered i) orally for five days (short-term exposure), ii) to adult animals, iii) at the lowest dose levels so far investigated, elicited sex-related effects in endocrine-active tissues such as thyroid, adrenal cortex, adrenal medulla and ovarian *granulosa* with changes in the serum levels of testosterone and T3 concurrently present, in the absence of general toxicity and with limited tissue deposition. A sex-related susceptibility was observed, with female reproductive, endocrine immune systems more affected (3).

Synthetic amorphous silica or SAS (SiO_2) is extensively used in food production as additive (E551). Nano-sized primary particles are formed in the production of E551 and then agglomerate later in the production process to larger structures [4]. Until now, nanosilica for food applications (Aerosil) has been produced but there is no information on the use, presence and concentration of nanosilica in food products also due to the insufficient regulatory frame at present.

Within the Joint Action NANOGENOTOX (http://www.nanogenotox.eu/) short-term oral exposure to SiO_2 nanoparticles (primary size ca. 20 nm) resulted in a limited absorption and deposition in liver and spleen of female rats. However, after IV (intravenous) administration, SiO_2 was still present in the organs at day 90 after the last treatment indicating biopersistence. The FP7 project NANoREG (http://nanoreg.eu/index.php) pursues a common European approach to the regulatory testing of manufactured NMs in order to provide tools for the definition of a credible and thorough nano-regulation. Within this project, NANOGENOTOX SAS study will be followed-up by performing a repeated-dose 90-day oral toxicity study in rat.

In conclusion, from this outline it is clear that further studies are required on oral exposure to TiO_2, SiO_2 and other NMs of relevance for present or future food applications. Risk assessment of NMs in food requires more data from long-term studies, at doses comparable with human intake *via* the diet and possibly focusing on susceptible populations and life-stages.

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RISK ASSESSMENT OF NANOMATERIALS BY THE EUROPEAN FOOD SAFETY AUTHORITY

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Nanomaterials have many potential applications in food/feed products. In Europe, various food/feed products require prior authorisation before being placed on the market, and the European Food Safety Authority (EFSA) is responsible for delivering scientific risk assessment opinions for this decision making process. A number of opinions have been delivered or are in the pipeline. Figure 1 shows the Scientific Panels which are currently involved in the assessment of nanomaterials along with the Scientific Committee.

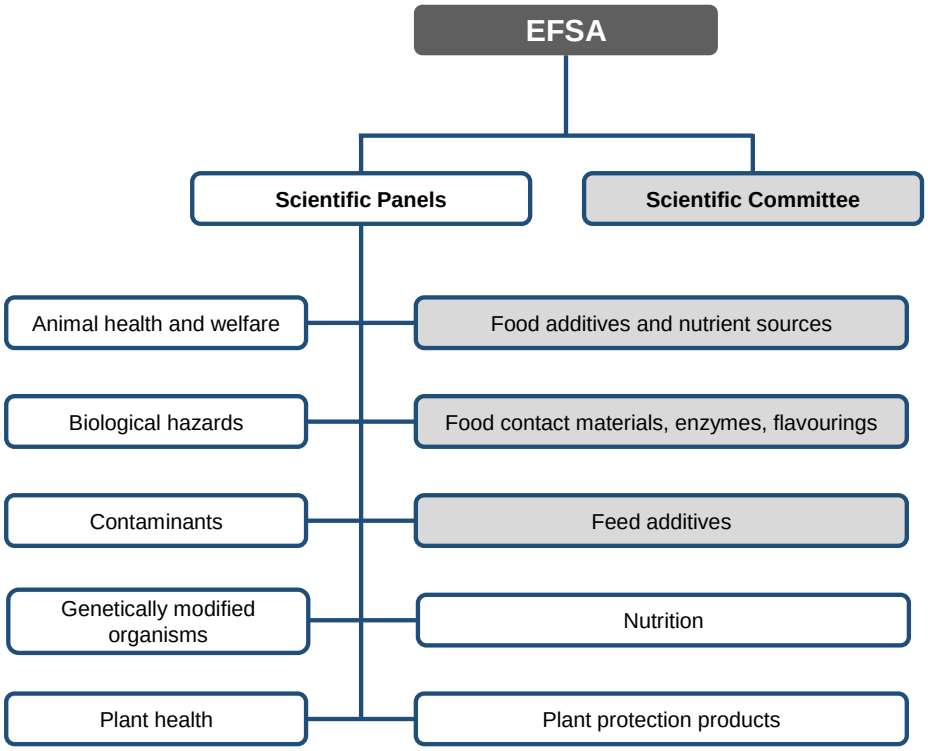

Figure 1. EFSA Scientific Panels and Scientific Committee (those currently involved in the assessment of nanomaterials are in grey)

In 2011 EFSA published detailed guidance on how nanomaterials in food/feed products should be assessed for human health risks (Figure 2) (1). This resulted not only in clearer and more transparent risk assessments by the concerned EFSA panels, but also in harmonisation when the same material was concerned for multiple food/feed purposes.

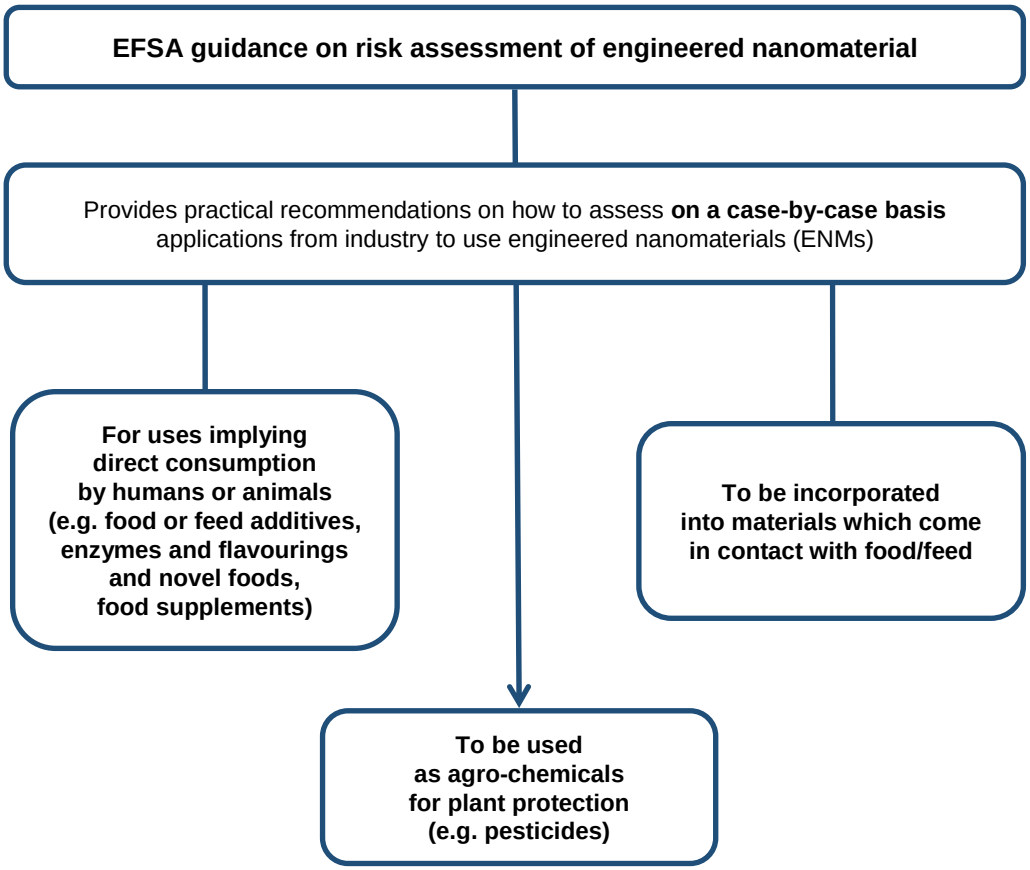

Figure 2. Three main categories of products/applications dealt with in the EFSA guidance on risk assessment of engineered nanomaterials

European Member States and other countries have welcomed this guidance and EFSA will provide updates as appropriate. New EU legislation for a definition and labelling of nanomaterial is expected and will feed into updating this guidance document. Also data of recent toxicological studies and test developments relevant for the oral route of exposure are useful for fine-tuning the risk assessment guidance.

To ensure good information exchange regarding data, expertise and risk assessment experiences, EFSA convenes on a yearly basis its Network for Risk Assessment of Nanotechnologies in Food and Feed (http://www.efsa.europa.eu/en/scnetworks.htm?wtrl=01). This network consists of delegates from the European Member States and pre-accession countries, delegates from the European Commission (e.g. DG Research&Innovation; Joint Research Centre, JRC) as well as EFSA staff, Panel Members and Scientific Committee Members.

The network has identified priorities and concentrated in its meetings on:

i) the recommended definition for nanomaterial (2);

ii) methods for detecting nanomaterial in complex matrices such as food/feed (e.g. those developed by the NanoLyse consortium) (www.nanolyse.eu);

iii) inventory lists of nanomaterial used in food/feed

iv) toxicological data (with relevance for food/feed) from national and European research projects.

In March 2013, EFSA awarded a contract for making inventory lists of nanomaterials being used in food/feed products. This information is collected from literature reviews and direct contact with applicants. Additional information on decisions for market authorisation, risk assessments or safety studies will be included in the database. These inventory lists are expected by March 2014 and will help to update the above mentioned EFSA risk assessment guidance. This database will also allow the EFSA Panels to anticipate the potential presence of nanoforms in their assessments and to check available information on the material of interest.

The activities of EFSA for nanomaterials help to ensure the safety of the European consumers. For the environment, risk assessments are carried out as well under the foreseen legal provisions.

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ACTIVITIES OF THE JOINT RESEARCH CENTRE OF THE EUROPEAN COMMISSION ON NANOTECHNOLOGIES RELATED TO THE FOOD SECTOR

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Nanoparticles are already used in several consumer products including food, food packaging and cosmetics and their detection and measurement in food represent a particularly difficult challenge (1).

In October 2011, the European Commission has published its recommendation on the definition of nanomaterial (2). This definition calls for the measurement of the number based particle size distribution in the 1-100 nm size range of all the primary particles present in the sample independently of whether they are in a free, unbound state or as part of an aggregate/agglomerate. This definition does present great technical challenges for developing measuring methods (3).

One important aspect of measuring the number-based nanoparticle size distribution are the difficulties involved in converting the mass or intensity based measurements to number based measurements. When weighted distributions are considered, the basis of the distribution is particularly important (Figure 1) and conversions between the different distributions are currently not very reliable. For instance, conversion requires information about particle shape that is assumed to be the same for all particles. This call for caution when data are evaluated and especially in using the conversion tools included in the software of most instruments.

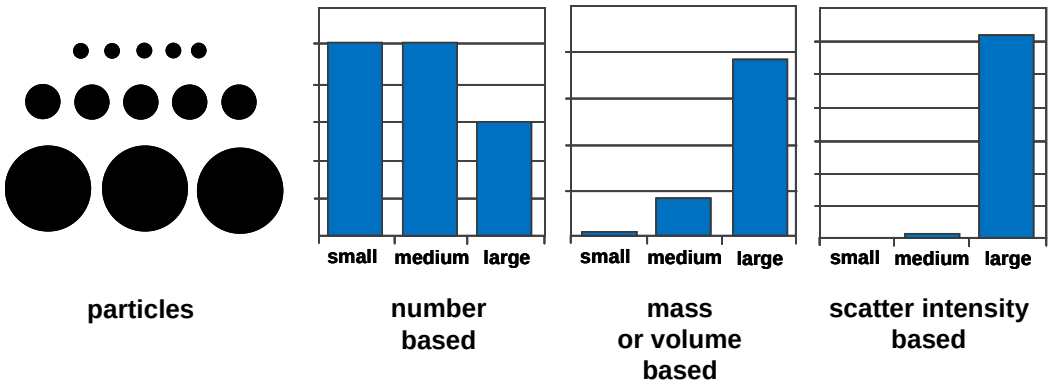

Figure 1. Number based, mass or volume based and scatter intensity based weighted distributions for a given number of particles of different sizes

The Joint Research Centre (JRC) of the European Commission provides scientific and technical support for the conception, development, implementation and monitoring of European Union policies. A brief overview on the current activities in the nanotechnology field is given in Figure 2.

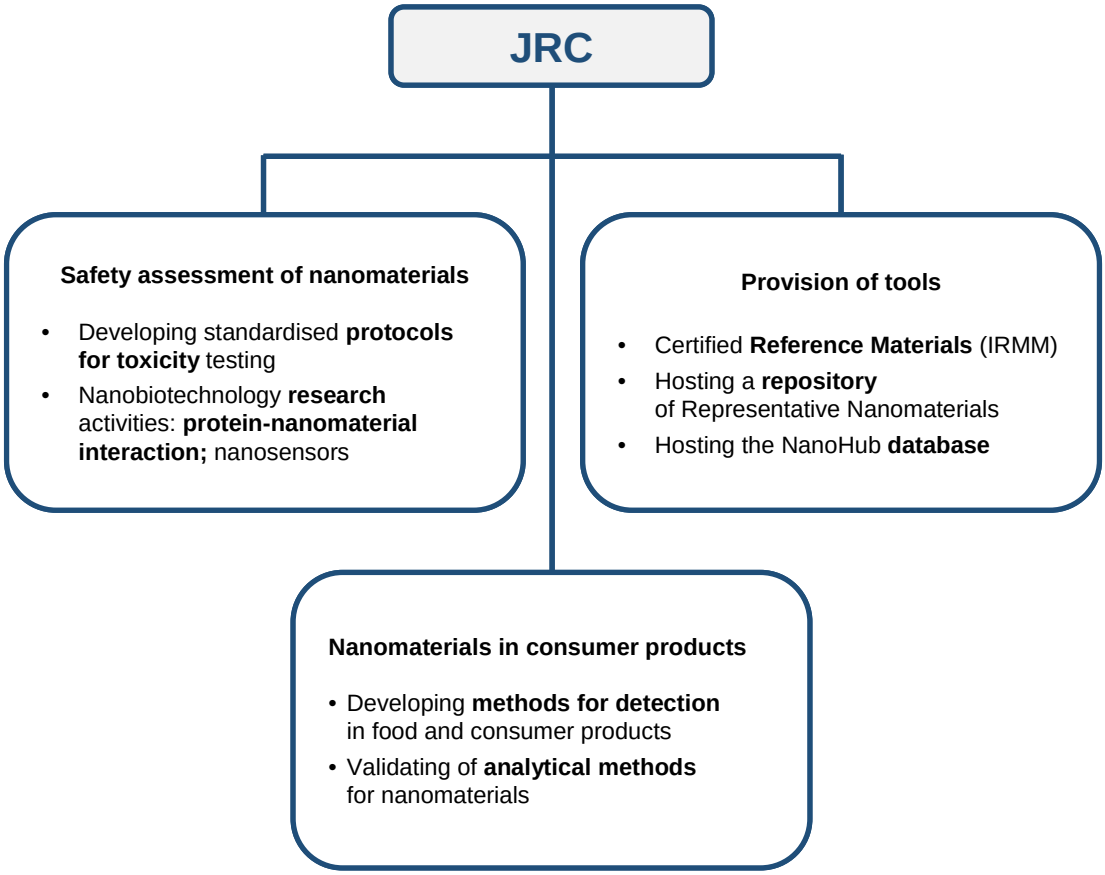

Figure 2. Current activities of the Joint Research Centre of the European Commission to support policy in the area of nanotechnology at the European Union level

When addressing the definition of nanomaterial of the European Commission, a critical aspect is the availability of techniques for the size measurement of nanoparticles. Existing techniques include different measurement approaches:

– *ex-situ*
  such as imaging methods based on Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Atomic Force Microscopy (AFM)
– *in-situ*
  such as light scattering methods based on static light scattering/MALS (Multi-Angle Light Scattering) , Dynamic Light Scattering (DLS), Particle Tracking Analysis (PTA)
– *pseudo in-situ*
  such as separation methods based on Field Flow Fractionation (FFF), Centrifugal Particle Sedimentation (CPS), Analytical Ultra-Centrifugation (AUC).

The requirements of an "ideal" method would be:
- particle specific;
- sensitive;
- able to determine number-size distribution;
- cost effective;
- robust;
- accurate;
- reproducible;
- fit-for-purpose.

At the moment no single method can satisfy all the requirements of the European Commission definition, therefore a compromise solution is required. However, new methods based on the combination of size separation techniques, such as flow field flow fractionation (4), with identification and quantification techniques, such as ICP-MS (Inductively Coupled Plasma-Mass Spectrometry), are particularly promising.

The problems to be overcome in measuring nanoparticles in food and consumer products are extremely challenging. There is a need to develop and validate tailored methods and reference standards (particles in relevant media) for specific applications. The interlaboratory performance study "detection/quantification of silver nanoparticles in an aqueous matrix" organized by JRC, which is currently in progress, is a first step in assessing the potential of FFF coupled with ICP-MS in this respect.

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PART 2
Additional materials

NANOMATERIALS IN THE FOOD SECTOR AND THEIR SAFETY ASSESSMENT: REFLECTIONS AND PERSPECTIVES

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The present contribution reports the main outcomes of the roundtable that concluded the conference "Nanomaterials in the Food Sector: New Approaches for Safety Assessment", organized by the Istituto Superiore di Sanità (ISS) and held at ISS on 27 September 2013. The roundtable, moderated by Francesco Cubadda and Alberto Mantovani (on behalf also of the other organizers of the conference), included the following participants:

- Luigi Calzolai, expert from the Institute for Health and Consumer Protection (European Commission - DG Joint Research Centre, Ispra);
- Stefano Pozzi Mucelli, expert from a research centre (European Centre for the Sustainable Impact of Nanotechnology – ECSIN Veneto Nanotech, Rovigo);
- Agostino Macrì, representative from a major national consumers' association (Unione Nazionale Consumatori, Rome).

The roundtable resulted in a lively debate, which involved the participants as well as the previous speakers and the audience at large.

The first issue presented for discussion was the foreseen relevance of the topic "nanomaterials in food" in the next decade from a food safety perspective. It was generally agreed that the application of nanotechnologies in the food sector, as well as in the feed sector, will attract increasing attention from all the actors of the food safety system in Europe and elsewhere. The involvement and the interest of risk assessors, risk managers, and/or laboratories in charge of official control will reflect and interact with the enforcement of sector-specific regulations. In their turn, technical and scientific bodies will get increasingly involved to meet the demands from regulators and risk assessors (e.g., to improve testing approaches or to reduce uncertainties). As soon as the nano-food field will have more perceivable impacts such as specific labelling or restrictions of certain products and media briefings and press coverage will intensify, interest by consumers' organizations will increase as well.

The second issue was the upcoming EU labelling regulation which prescribes that all ingredients present in the form of engineered nanomaterials have to be indicated in food labels as of December 2014 (1). The current proposal from the European Commission for the definition of nanomaterial, needed for implementing this regulation, sets that the fraction of nano-sized particles should be at or above the threshold of 50%. However, this provision does not cover food additives included in the "Union lists", i.e. permitted for use prior to the entry into force of Regulation (EU) No 1333/2008 after a review of their compliance with the provisions thereof. Regarding the proposed threshold, the main reason for selecting 50% appeared to be the technical feasibility of measurements based on available analytical detection methods. Noticeably, the current proposal states that the threshold may be replaced by one between 1% and 50% in the future, in light of technological developments concerning detection and quantification methods and where warranted by concerns for health and safety. This may be the case for some nanomaterials of interest in the food sector. The European Commission explains the exemption for the nanostructured food additives that are already on the market by the consideration that indicating them on food labels would have confused the consumers;

according to the European Commission, labelling would suggest that those additives are new while in reality they have been used in foods in that form for decades. This approach was criticized by many, especially by the representative of the consumers' association, since labelling should primarily inform consumers on what is in their food beyond any other considerations. From the standpoint of safety assessment, the discussion highlighted that these food additives were tested several decades ago, without consideration of their nano-sized nature and when the new conceptual framework of nanotoxicology simply did not exist. For instance, in the study reports of toxicity tests carried out at the time no characterization of the tested materials according to present standards can be found. Since all authorised additives are currently subject to a re-evaluation programme by the European Food Safety Authority (EFSA), covering also nano-related issues, such re-evaluation would be particularly important and urgent for some widely used nano-sized food additives. The discussion also highlighted the necessity for openness and transparency in risk assessment and communication and the need that EFSA maintains its current commitment to these core values.

The third topic was the importance of a comprehensive physicochemical characterization of engineered nanomaterials in the evaluation of their properties and safety; this requires the development and dissemination of specialized expertise. A robust and consistent characterization of nanomaterials will impact both risk assessment and risk management. For instance, it is unfeasible to classify as "monosubstance" manufactured nanomaterials (e.g. "synthetic amorphous silica", "titanium dioxide") that, in fact, are families including materials with different physico-chemical properties and biological activities. Advanced approaches such as grouping of nanomaterials based on the relationship between physico-chemical characteristics and toxicological properties and their "safe-by-design" production are promising; unfortunately, such approaches are not expected to have a tangible impact and any practical consequences in the short to medium-term.

Finally, the implications of the use of engineered nanomaterials in the agricultural production, as opposed to food processing and packaging, were discussed. In particular it was discussed whether the use of, such as nanopesticides or nano-sized feed additives may have any consequences in terms of users' exposure (e.g., farmers) or the environment. Overall, a consensus was reached on the fact that these issues are relevant and are expected to be increasingly significant in the near future. It was also noticed that the EFSA remit on pesticides as well as feeds involves an increasing attention to the assessment of potential risks for workers or the environment. Therefore, such issues as nanopesticides represent a field cross-cutting food toxicology and environmental toxicology: the risk assessment developments, therefore, might trigger interesting requirements for multidisciplinary research.

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REGULATORY ASPECTS RELATING TO NANOMATERIALS IN THE FOOD SECTOR IN THE EUROPEAN UNION

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The current legal framework in the European Union is made up by the horizontal legislation, the most typical example being the chemicals legislation (regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals, REACH; and regulation for Classification, Labelling and Packaging, CLP), and by the vertical legislation, which is sector specific (e.g., food and food contact materials regulations). The horizontal legislation is generally pre-nano and, for instance, only recently the REACH (Regulation (EC) No 1907/2006) has started to incorporate nano-specific provisions.

The vertical legislation in the food sector is more nano-specific, even though only in the case of Regulation (EU) No 1169/2011 on the provision of food information to consumers – or labelling regulation (1) – it includes a specific definition of the term "engineered nanomaterial" to enable specific provisions for nano-sized ingredients (in this case, the need of indicating them in the list of ingredients as 'nano') (Table 1).

Table 1. State of the art of the regulatory framework dealing with nanomaterials in the food sector

Legislation	Definition	Labelling	Specific provisions
Information to consumers	Yes	Yes	Labelling
Novel foods[a]	Yes	Yes	Separate assessment
Additives	No	No	Separate assessment
Food contact materials	No	No	Separate assessment

[a] This refers to the new draft regulation on Novel Foods

As discussed in other two contributions in this volume, the definition as it appears in the regulation itself is not consistent with the overarching definition of nanomaterial set forth in the Commission Recommendation 2011/696/EU and thus need to be adapted (*see* chapter "Labelling of nanomaterials in food: the Regulation (EU) No 1169/2011" in this volume), adaptation that is still underway with a proposal of the European Commission that has been the subject of criticism on some aspects (*see* chapter "Nanomaterials in the food sector and their safety assessment: reflections and perspectives"). The process has to be completed within the next few months since Regulation (EU) No 1169/2011 establishes that as of December 2014 all ingredients present in the form of engineered nanomaterials have to be indicated in food labels.

Another piece of the food sector specific legislation that has been dealt with in another contribution herein (*see* chapter "Engineered nanomaterials: authorization under novel food Regulation (EC) No 258/97") is the novel food regulation. By definition, food or food ingredients that result from the application of nanotechnology are considered *novel foods* since they originate from new production processes giving rise to significant changes in the composition or structure of food or food ingredients and pre-market authorisation is required for them (additives, flavourings or enzymes are not included since they are covered by separate

legal provisions). However, engineered nanomaterials are not explicitly mentioned in the current novel food regulation (2) whereas they will be in the upcoming Novel Food regulation that is presently under discussion. The new regulation will refer to the definition that will be adopted in the regulation on the provision of food information to consumers (see above) and, accordingly, the need for indicating the nano-ingredients in food labels will be reiterated (*see* Table 1).

The "nano" dimension also appears in the EU regulation on food additives (Regulation (EC) No 1333/2008) (3). The use of food additives in the EU is based on the principle that only additives that are explicitly authorised may be used in food. In addition, the quantities permitted are often limited and their use is in some cases restricted to specific foodstuffs. Under the law, food additives are defined as substances that are not normally consumed as food themselves, but are intentionally added to food for a technological purpose, such as food preservation. Prior to their authorisation by the Commission, food additives are evaluated for their safety by the European Food Safety Authority (EFSA). Regulation 1333/2008 also states that a new risk assessment by the EFSA is needed for new additives or already authorised additives (additives included in the "Union lists" established by Commission Regulations (EU) No 1129/2011 and (EU) No 1130/2011) when there is a change in particles size due to new production processes (*see* Table 1). Authorised food additives in the "Union lists" are subject to a re-evaluation programme by the EFSA also covering any nano-related issues (Commission Regulation (EU) No 257/2010). Examples of already authorised food additives containing a nano-sized fraction are E171, which is scheduled to be evaluated by 31.12.2015, and E551, which is scheduled to be evaluated by 31.12.2016.

Nanotechnology derived food packaging materials form the largest share of the current and short-term predicted market for nano-enabled products in the food sector. Main applications as Food Contact Materials (FCMs) include FCMs incorporating nanomaterials to improve packaging properties (flexibility, gas barrier properties, temperature/moisture stability), "active" FCMs that incorporate nanoparticles with antimicrobial or oxygen scavenging properties, "intelligent" food packaging incorporating nano-sensors to monitor and report the condition of the food, biodegradable polymer-nanomaterial composites.

Nanomaterials can be used in plastic FCMs only when they are explicitly authorised (Regulation (EU) No 10/2011), which requires a case by case evaluation from the EFSA (*see* Table 1) (4). At present there are three authorised nanostructured materials for use in plastic FCMs. Titanium nitride nanoparticles (FCM 807) can be used in polyethylene terephthalate (PET) up to 20 mg/kg under the condition of absence of migration. TiN agglomerates in the PET have a diameter of 100-500 nm consisting of primary nanoparticles that have a diameter of approximately 20 nm. Carbon black (FCM 411) is the second authorised material and consists of aggregates of a size of 100-1200 nm, originating from primary particles of 10-300 nm; the aggregates may form agglomerates within the size distribution of 300 nm to the mm size. The third authorised material is synthetic amorphous silicon dioxide (FCM 504), which consists of aggregated particles of a size of 0.1-1 μm originating from primary particles of 1-100 nm; the aggregates may form agglomerates within the size distribution of 0.3 μm to the mm size.

Regulation (EC) No 450/2009 on active and intelligent food contact materials describes nanomaterials as materials used in substances deliberately engineered to a particle size which exhibit functional physical and chemical properties that significantly differ from those at a larger scale (5). In line with the regulation on plastics it sets forth that nanomaterials need to be explicitly authorised. In addition, the nanomaterial that is released into food needs to be authorised as food additive under Regulation (EC) No 1333/2008. As an example, it can be mentioned the projected use of silver (Ag) nanoparticles in polyolefin food contact materials because of the silver antimicrobial effect. If the polyolefin article is designed to release silver

either as ion or as nanoparticle and it extends the shelf life of the food, then the polyolefin food contact material would be considered as an active material or article according to Regulation (EC) No 450/2009 and should be assessed and authorised as such.

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ANALYTICAL DETERMINATION OF ENGINEERED INORGANIC NANOMATERIALS IN FOOD AT THE ISTITUTO SUPERIORE DI SANITÀ: INTERLABORATORY STUDIES

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In 2011 the European Commission, in response to a request from the European Parliament calling for the introduction of comprehensive a scientific-based definition on nanomaterials in the Union legislation, adopted the Recommendation 2011/696/EU (1) setting out a definition for determining whether a material should be considered as a 'nanomaterial' or not for legislative and policy purposes.

The definition requires the materials to be characterized in terms of the number size distribution of their constituent particles and that reliable measurement methods are available. Implementation of this definition within the context of legislative controls will require improvement of current methods and development of new and fit-for purpose ones. Therefore, to address the analytical challenges and to provide valid technical input to potential legislators, interlaboratory programmes have been undertaken with the aim of validating testing protocols.

As far as nanomaterials in food are concerned, three interlaboratory studies have been carried out by the European Commission so far on the determination of silver nanoparticles (AgNPs) in food simulants/matrices:

- two of these studies based on single-particle Inductively Coupled Plasma-Mass Spectrometry (sp-ICP-MS) (named "Interlaboratory method performance study for the determination of Ag nanoparticles by single particle ICP-MS");
- one study based on Asymmetric Flow Field Flow Fractionation (AF4) coupled to ICP-MS (AF4-ICP-MS) (named "Determination of Ag nanoparticles by Identification and quantification of Ag nanoparticles in aqueous suspensions combining Asymmetric Flow Field Flow Fractionation and Inductively Coupled Plasma-Mass Spectrometry (AF4-ICP-MS").

Among the participants recruited from research, industry and regulators, only few laboratories worldwide were involved in all of the three exercises, the team dealing with food NMs at the Department of Food Safety and Veterinary Public Health of the Istituto Superiore di Sanità (the National Health Institute in Italy) being one of them.

Determination of AgNPs by sp-ICP-MS

A promising tool for the detection and quantification of inorganic nanoparticles is sp-ICP-MS. For a broader application of the sp-ICP-MS technique it is essential to demonstrate its transferability to other laboratories. To this end, the RIKILT Institute of Food Safety (The Netherland) organised an interlaboratory method performance study entailing sp-ICP-MS as the analytical technique, in collaboration with IRMM (Institute for Reference Materials and Measurements – European Commission, Joint Research Centre, Geel, Belgium). The main goal of the study was the evaluation of precision and trueness of the size determination using sp-ICP-

MS in the size range of particle diameters from 20 to 100 nm, under interlaboratory conditions. Also the precision of the particle number concentration was evaluated. The study involved 23 selected laboratories from Europe, the USA and Canada recruited in universities, research centres and instrument manufacturers. AgNPs were selected as target analyte for the first as well as the subsequent validation studies of sp-ICP-MS for the sizing and quantification of nanoparticles in aqueous media (2, 3) and complex matrices, respectively.

Principle of the method

The detection by sp-ICP-MS involves the use of suspensions sufficiently diluted so that most droplets entering the plasma do not contain any particle and the probability of having two or more particles in one droplet can be neglected. In the plasma, particles are vaporised and the individual atoms are ionised resulting in a cloud of ions. This cloud of ions is sampled by the mass spectrometer and detected as a signal pulse in the detector. If not more than one nanoparticle is introduced into the ICP during the reading time, the number of counts of each recorded pulse is related to the quantity of atoms in the particle, and the frequency of the pulses is proportional to the number concentration of nanoparticles. By plotting the number of nanoparticles detected producing a signal of i_{NP} counts *versus* i_{NP}, a signal distribution is obtained, which can be related to the nanoparticle size distribution. The intensity of the signal pulse is directly proportional to the mass of the detected nanoparticle, and thereby to the nanoparticle's diameter to the third power (i.e., assuming a spherical geometry for the nanoparticle). As well as nanoparticle size distributions, the average nanoparticle number concentration of a suspension and the mean diameter can be estimated (4). A typical analysis time is 60 seconds and is called a time scan. The mass spectrometer can be tuned to measure any specific element, but due to the high time resolution needed only one *m/z* value can be monitored during each run.

First round: determination in suspensions

Two standard food simulants, namely, de-ionised water and 10 % v/v ethanol, were selected as test matrixes for this first intercomparison. Test samples consisted of spherical monodisperse AgNPs suspensions in water with approximate Ag mass fractions ranging 13-25 mg/L and covering the 20-100 nm size range, along with a blank sample. Detailed Standard Operating Procedures (SOP) were provided to participants that were also asked to dilute the suspensions in order to obtain 8 preparations to be analyzed by sp-ICP-MS. Each of the eight suspensions was analysed in triplicate for Ag mass fraction and particle size of the AgNPs assuming perfectly spherical particles, ignoring adsorbed stabilisers.

Due to lack of suitable reference materials, calibration was performed using ionic silver standard solutions analyzed under the same conditions. The nebulisation efficiency was determined by gold nanoparticles reference material, NIST 8013, consisting of a suspension of gold nanoparticles with a mass concentration of 50 mg/L stabilized in a citrate buffer. These particles have a spherical shape and a diameter of 60 nm. Currently, no commercial software for the evaluation of sp-ICP-MS is available therefore the organizers provided the participants with a dedicated Excel spreadsheet. The data were exported as a CSV file and imported in Excel to calculate the number and mass concentration, and the size and size distribution of the nanoparticles.

Second round: determination in chicken meat

At the end of May 2013, participants of the first intercomparison were invited to take part to the second interlaboratory study on sp-ICP-MS. For this exercise, a real food sample, i.e. chicken meat, had to be analysed for nano-Ag particles above 20 nm since the first study on aqueous suspensions showed that it was still too demanding for most of laboratories to determine particles with a diameter of ca. 20 nm.

Six sample vials were delivered to participants: two blank chicken homogenates, two vials of chicken homogenate spiked at level 1 (approximately 0.1 g/kg) and two vials of chicken homogenate spiked at level 2 (approximately 0.5 g/kg) (Figure 1). As for the first round, SOPs were provided for the sp-ICP-MS analyses and for the sample preparation of the chicken sample, consisting in a enzymatic matrix degradation. The enzyme to be used for sample enzymatic extraction (proteinase K), was shipped with the samples. In order to cover the scope of the intercomparison, participants were also asked to prepare and measure spiked control samples with a known standard, namely EAW 1093 60 nm silver nanoparticles from NanoComposix (San Diego, CA).

Figure 1. Blank and AgNPs spiked chicken homogenates delivered within the second round on sp-ICP-MS

Results and conclusions

The first interlaboratory exercise was concluded in February 2013 with the delivery of the final report to the participants. Figure 2 shows a sequence of sp-ICP-MS data processing obtained in the authors' laboratory for an aqueous suspension of AgNPs. From the time scan (Figure 2a), the Ag signal was plotted as a frequency distribution (Figure 2b) and visually inspected in order to select the pulses resulting from NPs and distinguishable from the background (e.g., instrumental noise, ionic silver, etc.). Assuming a spherical particle shape and knowing the chemical composition of the particles (Ag), it was thus possible to calculate the diameter of the particle (Figure 2c) and gather the descriptive statistic to fulfil the EC Recommendation (number-based).

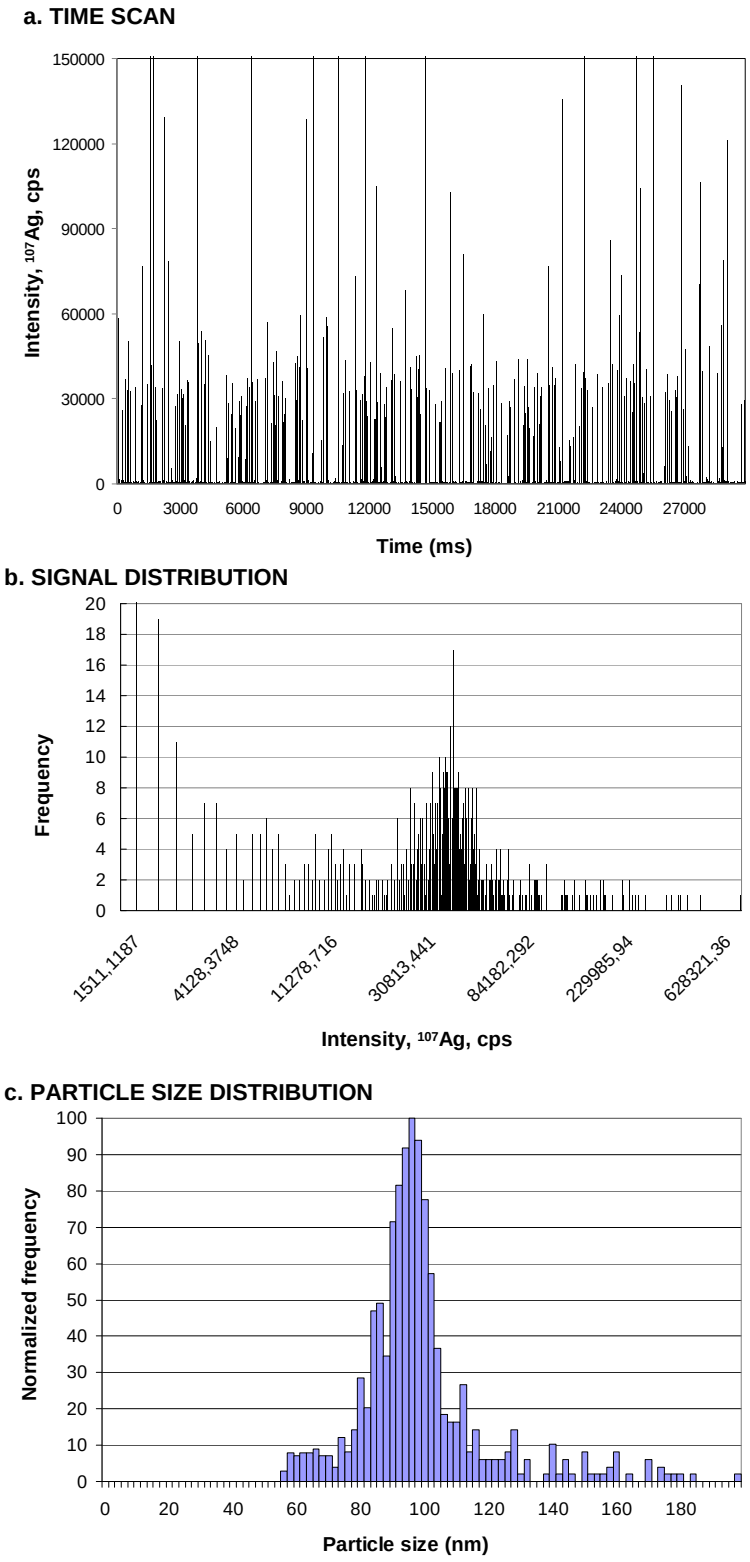

Figure 2. sp-ICP-MS analysis and data processing obtained at the ISS for AgNPs: intensity *vs* time plot (a); frequency distribution plot of measured intensities (b); size distribution plot for suspension 3 (c)

The general outcome of the first round was satisfactory. In summary, taking into consideration the novelty of the method, the fact that the vast majority of participants had just started establishing the method in their laboratories and the lack of suitable certified reference materials for calibration, the accuracy of sp-ICP-MS is already satisfactory for screening purposes, at least limited to particle diameter. In its current state, the method can only approximately quantify the amount of nanoparticles (mass or particle number concentration) (3). The second round is still in progress and is expected to give the first outcomes by the end of 2014.

As a general remark, while further improvements are certainly desirable (especially with respect to software tools for data treatment, hardware options for shorter dwell times, calibration standards for determining nebuliser efficiency and further experience by laboratories), the results of this studies shall demonstrate the suitability of sp-ICP-MS for the detection and quantification of at least some important types of inorganic nanoparticles.

Determination of AgNPs by AF4-ICP-MS

The Institute for Health and Consumer Protection (IHCP) of the Joint Research Centre (JRC) of Ispra (Italy) coordinated the first validation study on the detection and quantification of particles. The exercise focused on AgNPs suspended in a simple aqueous matrix, being those particles considered as a priority by the European Commission. In March 2013, expert laboratories were invited to participate in an interlaboratory comparison undertaken for the evaluation of an analytical method for determining nanoparticle number-size distribution by means of AF4-ICP-MS.

The collaborative study consisted in three steps (two stages of experimental work with an intermediate workshop):

- *Stage 1 (initial study)*
  This stage correctly established the test in the participant laboratories (March 2013). It is intended as a training/learning exercise in which the participants are supplied with a series of simple mono-modal nanoparticle dispersions with declared sizes and approximate concentrations.
- *Workshop*
  Based on the results of the first phase, a training and evaluation workshop will be organized by JRC in which the result will be presented and if necessary on-site training provided.
- *Stage 2 (ring trial)*
  Each laboratory will be supplied with a standard operation procedure for the analysis procedure together with identical blind samples and any necessary calibration or reference materials. The outcome of the analysis will be used to assess the performance characteristics of the test.

Principle of the method

The untreated sample is injected in the sample loop of an AF4 system and subjected to size fraction separation, which results in smaller particles eluting before larger ones (range 10-100 nm). For detection and quantification of the size fractions of inorganic NPs, the AF4 system is coupled online to an ICP-MS instrument. An UV/VIS (Ultraviolet/Visible) detector is used as

auxiliary detector for the preparatory steps of method optimization and later on as additional detector in combination with ICP-MS.

When analysing unknown Ag colloidal samples the size of nanoparticles is determined by calibrating the detection system for size against elution time using nearly mono-dispersed AgNPs standards supplied by the organizers. For the quantification of silver mass, the ICP-MS instrument response must be calibrated using appropriate silver reference solutions. This stage may be approached using methods already established in the participant's laboratories or alternatively by flow injection of ionic silver standard solution.

Stage 1: correct application of the method in participants' laboratories (method familiarization)

Each participant laboratory was asked to optimize the instrumental conditions for its own combination of equipment (AF4, UV/VIS detector, ICP-MS) to get familiar either with the method developed by the JRC and the detection and quantification of mixtures of nanoparticles. The method was turned into simplified SOP and tested with mono-modal (nearly mono-dispersed) samples of declared size and concentration supplied by the organizers. This first stage study concentrated on evaluating the instrumental analysis aspects of determining the number size distribution of AgNPs suspended in a simple aqueous matrix. Figure 3 shows the scheme to be followed according to the provided SOP.

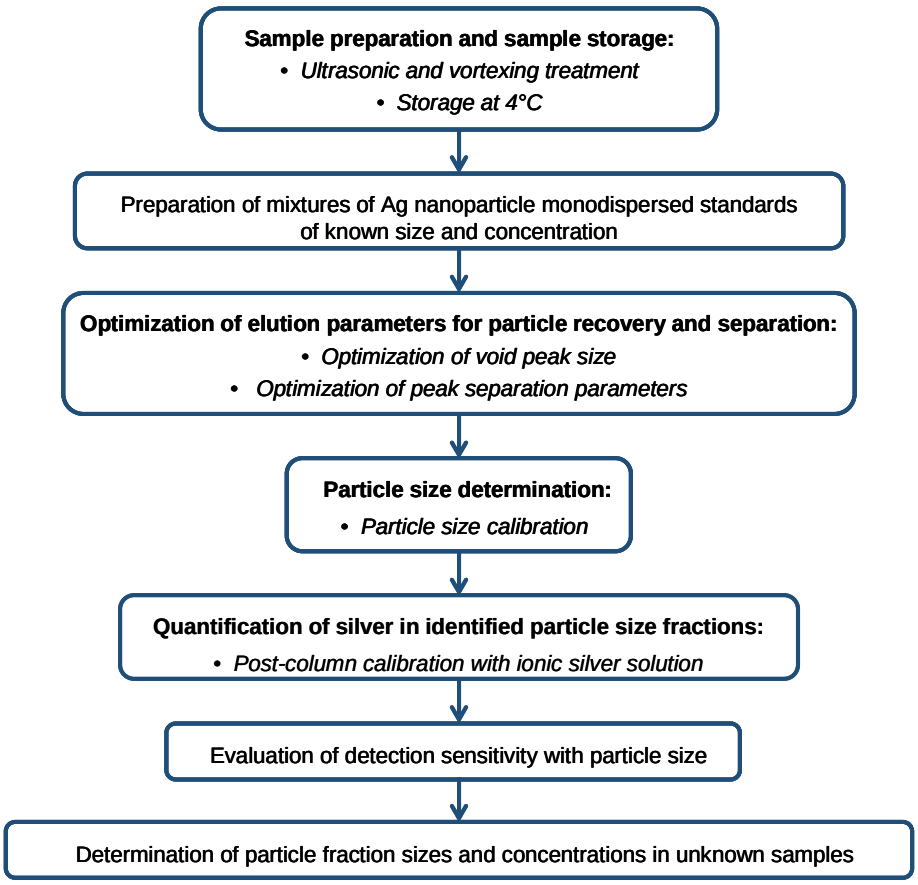

Figure 3. Scheme for the correct application of the AF4-ICP-MS method

Results and conclusions

An example of the separation under optimized conditions obtained at the authors' laboratory for a AgNPs mixture (10/40/100 nm) coupling AF4 with both UV and ICP-MS detectors is given in Figure 4. Phase 1 of the study is in progress, being the results collected from participating laboratories still under evaluation by the JRC. The results obtained from the unknown materials will be used to evaluate the performance of the test method in the different laboratories and, if positive, move to the next stage of the intercomparison.

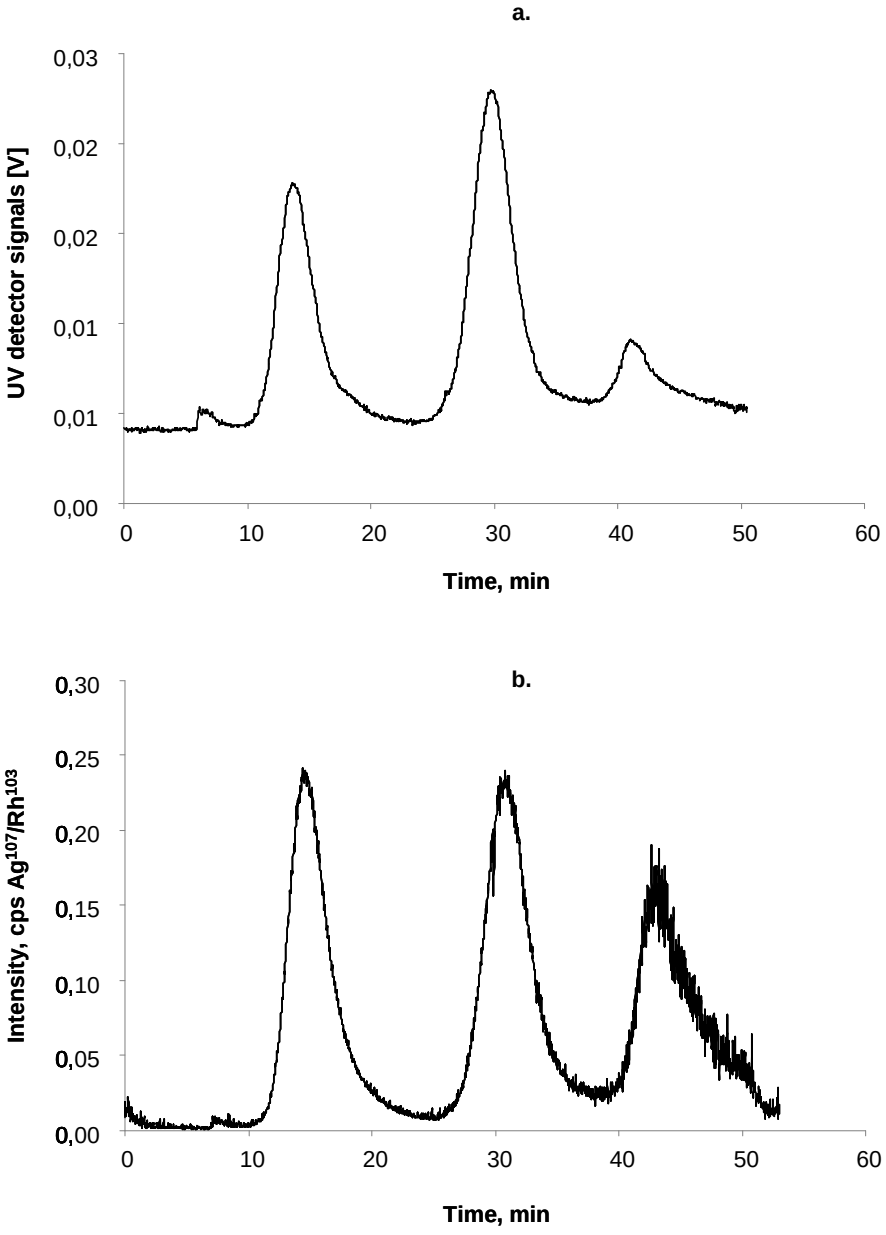

Figure 4. Fractogram example of an AgNPs 10/40/100 mixture coupled to UV/VIS (a) and ICP-MS (b) detectors

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