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Asymmetric Flow Field Flow Fractionation (AF4) and Inductively Coupled Plasma- Mass Spectrometry (ICP-MS) methods as a means to determine nanoparticle number size distributions of silver nanoparticle dispersions

Report of stage 1 of inter-laboratory trial

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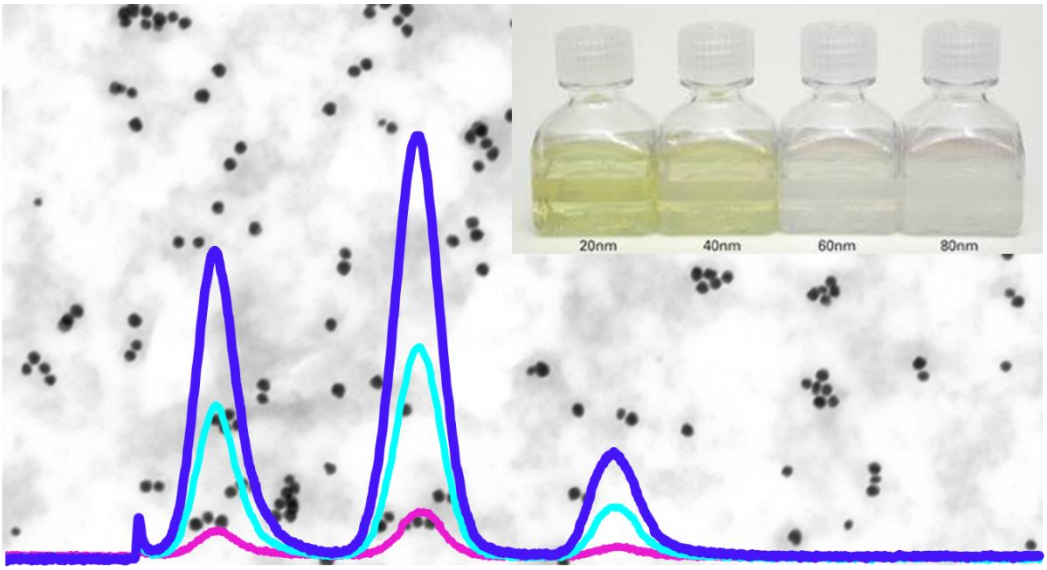

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Cover: Transmission electron micrograph (TEM) images of silver nanoparticles overlaid with AF4 fractogram

In support of the implementation of the European Commission recommended definition of a nanomaterial the Institute for Health and Consumer Protection of the Joint Research Centre of the European Commission has been developing potential methods for measuring nanoparticle number size distributions.

As part of this activity a method which combines a particle size separation (Asymmetric Flow Field Flow Fractionation (AF4)) step and a particle detection/quantification step (Induction Coupled Plasma-Mass Spectrometry (ICP-MS)) has been examined and optimised for the analysis of aqueous dispersed silver nanoparticles. Following an internal validation, the method has been documented in the form of a Standard Operating Procedure (SOP) designed to provide all the necessary information to allow the method to be applied by suitably equipped external laboratories. To verify the transferability of the method an international ring-trial was organized by JRC in which 8 independent laboratories were provided with detailed documentation and suitable test materials to allow them to test the transferability of the SOP.

This report details the organization of the trial, presents the experimental results obtained and summarises the conclusions and recommendation coming from a detailed consideration of the data obtained.

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In the following report it is intended that the data reported be anonymous and consequently the list of participating laboratories in the table above is given in alphabetical order of identifier which does not correspond with the order in the rest of the report.

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Executive Summary

Implementation of the European Commission recommended definition of a nanomaterial within the context of legislative controls requires that enforcement laboratories be provided with fit-for-purpose analytical methods. Currently no suitable validated analytical methods are available for this purpose.

To address the analytical challenges and to provide valid technical input to potential legislators JRC-IHCP has, on specific request from DG-SANCO, begun a study program aimed at the development of methods which can be applied to measuring the number size distribution as required for the technical implementation of the common definition for the term nanomaterial.

As part of this activity a detailed study was undertaken by JRC to develop specific methods for the analysis of aqueous dispersed nanoparticles. After careful evaluation of a range of possible analysis techniques, development work was started on a method which combines particle size separation by Asymmetric Flow Field Flow Fractionation (AF4) step with a particle detection/quantification step based on Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). The simplified method requires the use of pseudo-standards to calibrate particles size and concentration. These two methods together allow the measurement of a particle mass-size distribution which may, assuming the particles are near spherical, be mathematically converted into a number-size distribution as required by the EU definition.

This general method is being studied for use with a number of high priority nano-materials and in particular has undergone detailed development for use with one specific type of silver nanoparticle dispersion. At this time the method for detection and quantification has now been developed by JRC into a simplified Standard Operating Procedure (SOP) which has been used in the first stage of a small scale inter-laboratory comparison.

The scope of this study was to evaluate whether the basic particle separation methodology could be transferred from the development laboratory (JRC) to the other participant laboratories in the trial. To assist in the method transfer a series of pseudo-standards composed of solution of near mono-dispersed silver nanoparticles with known size and concentration were supplied to the participant laboratories. These materials, when used singly or prepared as appropriate mixtures, were to be used to optimize and verify the efficiency of the separation methodology when applied in each participant laboratory.

As a verification of the optimized separation the participants were also supplied with two unknown samples (A and B) which were to be analysed with no prior knowledge of their composition. These samples were made by combining appropriated quantities of the 50nm and 100nm dispersions so as to produce a mixture in which the number of 50nm and 100nm particles was theoretically equal.

This particular mixture was chosen as this represent the borderline case between a nanomaterial and a non-nanomaterial under the criteria of the EU recommended definition. The following report will detail the results and present conclusions on the outcome of the study. The results obtained have led to the following main conclusions

1) The separation methodology, when applied with the reference standards was found to be transferable to different laboratories with all laboratories being able to separate the recommended mixtures of mono-dispersed materials.

2) In the course of the study it was found that two laboratories reported problems of separation resulting from the quality of the separation membranes. Substitution of membrane with that from alternate batches or alternate manufacturers was found to resolve the problem. It should be noted that during method development this problem has also been observed by the organizing laboratory (JRC) although during the laboratory trial the problem was not observed.

3) The separation methodology, when applied with the "unknown samples A and B" and using UV absorption detection showed five of nine laboratories as being able to clearly separate the bimodal mixture. Three of the nine laboratories were able to obtain elution curves which showed evidence of the two peaks but with poor signal/noise ratio. Only one laboratory was not able to show evidence of the bimodal mix.

4) A statistical analyses of the result obtained from the unknown samples showed that acceptable accuracy and reproducibility was obtained for the measurement of nanoparticle size but that the quantification of the amount of silver using the ICP-MS was not sufficiently accurate or reproducible.

5) Sample stability: It is known that silver nanoparticles often exhibit problems of stability and may suffer from aggregation or dissolution during long term storage. A number of the participants were not able to complete their analyses in the recommended time period and it is likely that this may have influenced negatively on the quality of results obtained.

Glossary

AgNP	Silver nanoparticles
AgNP10, AgNP20…	Silver nanoparticles of nominal diameter of 10 nm, 20 nm…
AF4	Asymmetric-Flow-Field-Flow-Fractionation
Agglomerate	Collection of weakly bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components.
Aggregates	Particle comprising strongly bonded or fused particles
CLS	Centrifugal Liquid Sedimentation
CPS	Counts Per Second (ICP-MS signal output)
DLS	Dynamic Light Scattering
EM	Electron Microscopy
FFF	Flow Field Fractionation
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	Inductively Coupled Optical Emission Spectroscopy
MALS	Multi-angle light scattering
Particle	A minute piece of matter with defined physical boundaries;
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
ILC	Inter-laboratory comparison
CAT	Chemical Assessment and Testing unit
NBS	NanoBioSciences Unit
SOP	Standard Operation Procedure

1 Introduction

In 2011 the European Commission, in response to a request from the European parliament, agreed on a common definition for the term nanomaterial which requires that materials be characterized in terms of the number size distribution of their constituent particles[1][2]. Implementation of this definition within the context of legislative controls requires that enforcement laboratories be provided with fit-for purpose analytical methods.

To address the analytical challenges and to provide valid technical input to potential legislators JRC-IHCP has, on specific request from DG-SANCO, began a study program aimed at the development of methods which can be applied to the technical implementation of the above noted definition. The outcome of this study would, ideally, result in the publication of a validated test protocol applicable to at least one technically relevant type of liquid dispersed nanoparticle.

As part of this activity a detailed study was undertaken by JRC to develop a specific method for the analysis of aqueous dispersed silver nanoparticles by use of combined Asymmetric Field Flow Fractionation (AF4) and Induction Coupled Plasma-Mass Spectrometry (ICP-MS). This method has now been developed into a simplified Standard Operating Procedure(SOP) which is reported in Annex 5 of this document. The further development of this procedure towards becoming a validated test protocol now requires the method be evaluated by inter-laboratory ring-trials.

Given the technical complexity of the problem, it was decided that the process of validating the SOP through inter-laboratory studies should be split in two stages: a method familiarization stage (Stage 1) and an eventual validation study (Stage 2). This document will detail the results obtained by the participants in stage 1 of this process.

The first stage of this study was to assess whether the analysis procedures developed by JRC can be successfully transferred to other independent laboratories equipped with comparable but not necessarily identical AF4-ICP-MS facilities. It was foreseen that the methodology be usable by laboratories with existing experience of AF4 but not necessarily with experience of the nanoparticle mixtures of the type being examined here. It was, therefore, necessary that each laboratory optimize the instrumental conditions for their own combination of equipment using a series of mono-modal (near mono-dispersed) samples of declared size and concentration supplied by JRC. For an evaluation of the final optimized conditions simple bi/tri-modal samples of undeclared size and concentration were supplied for analysis.

2 Materials and methods developed for the inter-laboratory comparison

As a first step in addressing the analytical challenges of the definition an experimental study was initiated within JRC-IHCP whose scope was to identify flexible methods for the analysis of nanoparticle size distributions. In identifying suitable methods consideration was given not only to technical issues but the urgency to find possible methods and procedures which would be as cost effective as possible. The following section details the considerations given in the choice of the method and analyte particle together with a description of the chosen method itself.

2.1 Choice of analytical method for study

Currently there is no single analytical method able to satisfy the requirement of the definition but a variety of methods exists which could be applied in the resolving parts of problem. A detailed overview of the area and has been prepared by JRC and published in series of reference reports [3] [4], [5]. In particular, this first of these reviews [3] provides a comprehensive overview of common methods with descriptions of their operational principles and their advantages and disadvantages in regard to the making measurements of nanoparticle number size distributions.

After a detailed consideration of the possible methods available the combination which was thought most likely to be able to satisfy, at least in part, the requirement of the definition for the largest possible range of materials and concentrations was Asymmetric Flow Field Fractionation (AF4) separation combined with on-line elemental detection by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). This combination permits the fractionation of poly-dispersed mixtures as a function of their size and then quantifies the mass of particulates in those fractions by use the high sensitivity element specific ICP-MS. Provided that a suitable method is available to determine the mean particle size in the eluted fractions a mass-size distribution may be generated and then, assuming the particles are near spherical, be mathematically converted into a number-size distribution as required by the EU definition. The resulting mass-size distribution technique of designed to allow calculations of number size distribution from an experimentally determined mass size distribution as follows. The following section will describe the following.

2.1.1 Field flow fractionation (FFF)

Field-Flow Fractionation[6] is actually a family of separation techniques, comprising various different sub-techniques which all utilize the same basic separation principle but employ different force fields. Depending on the type of separation field used the technique may be called Flow Field-Flow Fractionation, Sedimentation Field-Flow Fractionation, Thermal Field-Flow Fractionation or Split Flow Thin Cell Fractionation (SPLITT). The separation process is similar to chromatography except

that no solid phase is used and the separation is based on applying a physical force to the particles in solution as opposed to a chemical interaction as shown in Figure 1.

Most variants of FFF perform their separation in a thin ribbon like channel of liquid which flows between two flat plates separated by a thin spacer foil of typically 100-500 µm thickness. In such a narrow channel the liquid moves under laminar flow conditions which means that the velocity of the liquid varies with the distance from the channel wall, slowest at the wall and fastest in the centre of the channel. When a perpendicular separating force is applied to the channel the particles will move toward the lower wall while the natural Brownian motion in solution will tend to counteract this effect allowing particles to diffuse back toward the centre of the channel. When these two forces reach an equilibrium the particles become distributed across the channel with the smaller, faster diffusing particles tending to pass more time on the high flow region while the larger slower diffusing particle remain more in the slower flow stream near the wall.

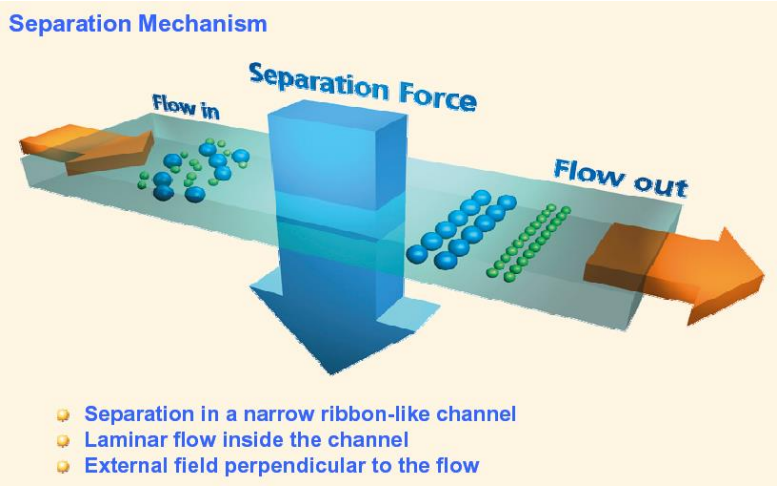

Figure 1 General principle of FFF operation[1]

Image: Postnova Analytics GmbH: **http://www.postnova.com/general-theory.html**)

The overall effect is that as the particles flow along the channel, smaller particle will move faster and be eluted from the column before larger slower moving particles. In the majority of FFF methods the particle separation is based principally on their hydrodynamic size or, in the case of Sedimentation Field-Flow Fractionation, a combination of hydrodynamic size and particle density.

In this work the most flexible and commonly used version of FFF, Asymmetric Flow Field Flow Fractionation (AF4), has been chosen for method development. The AF4 method utilises a very specific channel geometry which is shown in schematically in Figure 2.

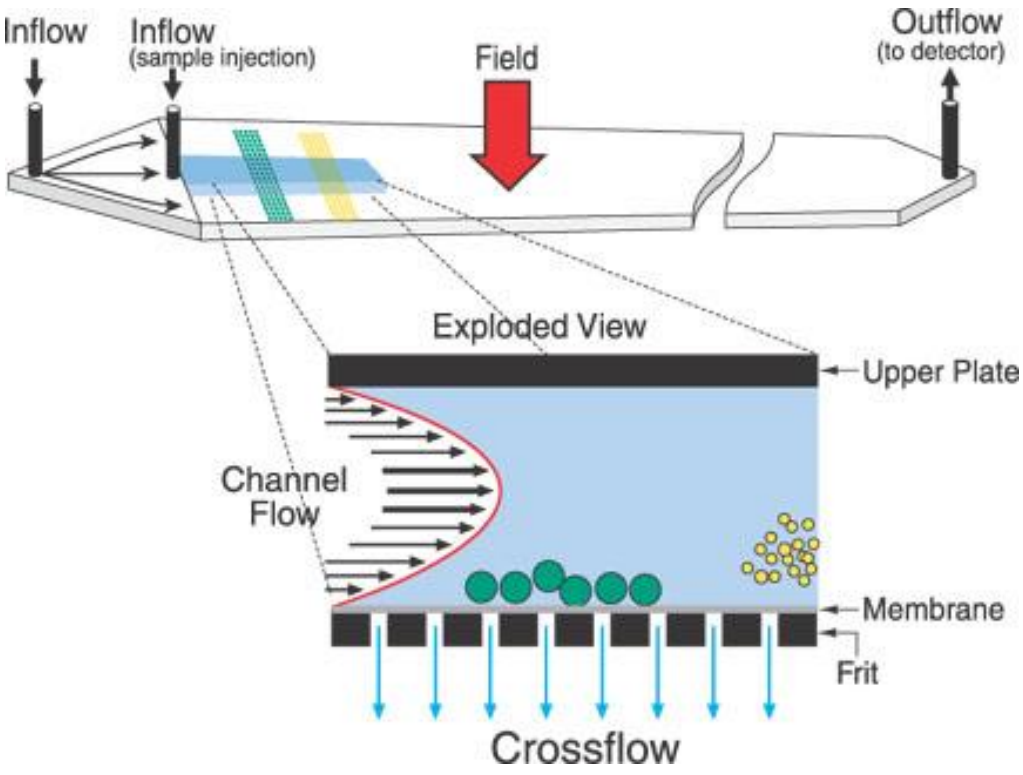

Figure 2 Schematic of Asymmetric Flow Field Flow Fractionation(AF4) channel

Image: Wyatt Technology: http://www.wyatt.com/library/theory/flow-field-flow-fractionation-theory.html

As can be seen the AF4 channel is formed by a thin spacer foil which separates an impermeable upper wall from a lower semipermeable membrane supported by a porous frit. During the particle separation phase of operation a flow of eluent is pumped through the AF4 channel generating a laminar flow velocity profile while a second pump simultaneously extracts a portion of the liquid across the membrane and frit. The resulting tangential flow of liquid, known as the cross flow, provides the separating force which pushes the particles towards the semi-permeable membrane.

The membrane used in AF4 may have various molecular cut-of values, ranging from as low as 300Dalton to more than 100kDalton depending on the sample type which has to be fractionated. The membrane used needs to have a pore size which is sufficiently small as to retain the particles within the channel while still allowing the flow of liquid without excessive pressure drop. For the separation of typical nano-particulate materials membranes with a 10kD cut-off provide a good compromise for effective retention of particulates with a moderate pressure drop. The AF4 method can be applied to the fractionation of particles in the range from around 1nm to over 500nm and therefore is broadly suitable for application to the EC definition.

2.2 Choice of detection and size/mass quantification method

It is important to note that FFF is, in reality, a separation technique, not a size measurement technique and the FFF column must normally be coupled to a detector system that performs on-

line/off-line detection, quantification and/or sizing. In some cases quantification of the mass/concentration of particles may be achieved using moderately priced on-line detectors such as UV-Visible light absorption or refractive index with the actual applicability of the method being dependant of the type of particles. In other cases, the levels of sensitivity necessary may require more costly and sophisticated element specific methods such Inductively Coupled Plasma Mass Spectrometry (ICP-MS) which is a highly sensitive analytical technique which uses mass spectrometry for the detection of metals and several non-metals in liquid samples at concentrations as low as one part in 10^{12} (part per trillion) for batch analysis. For the measurement of size, static (MALS) and dynamic light scattering (DLS) methods may be applied on-line although such techniques again add significant cost and may not be able to give the desired information due to key technical limitations. For example, MALS may be sufficiently sensitive to detect the presence of small particles but the physical basis of the measurement impose limits on the lower size of particles which can be sized. In the case of DLS the lower size does not have the same intrinsic limitation but instead may be unable to operate because of the low scattered light signal which is achievable with small particles at the concentration levels typical of analytical FFF.

In the situation where issues of sensitivity or cost make the use of direct on-line sizing undesirable there remain a number of other alternatives which can be considered. One of the simplest strategies for sizing and quantifying the materials eluted from an FFF system is to do time resolved collection of sample aliquots and then do off-line analysis of each sample to determine the size and concentration. This strategy offers the advantage of being able to analyse the samples under optimised conditions while also allowing the analysis instrumentation to remain independent of the FFF systems. This would be relevant in laboratories where high cost polyvalent analytical instrument such as ICP-MS are available but cannot be dedicated exclusively to use with FFF. The disadvantage of the off-line analysis method is that, depending in the sampling time, the time/size resolution may be reduced and in most cases will greatly increase the time necessary to generate a full data set of a particle size distribution. In this strategy particle size could be determined off-line using a variety of methods including DLS, MALS and electron-microscopy.

In the absence of any other instrumental methods the particle size can be calculated from the experimentally measured values of retention time following application of the established, basic theory of FFF. It should be noted that this theory generally assumes "ideal" separation conditions and therefore no significant interaction between particles and membrane and this is cannot always guaranteed particularly when examining particle with unknown or poorly defined surface chemistry.

In a variation of this strategy retention time can be used to determine particles size by use of experimentally determined calibration curves of size and retention time obtained using (certified)

particle size standards. Ideally this should be done using particles which are chemically similar to those expected in the unknown samples as variations in surface chemistry may modify retention times as a result of different particle-membrane interactions. If chemically identical calibration standards are not available then alternatives such as size certified latex particles may be considered provided that eluent and elution conditions can be determined which minimise particle-membrane interactions.

2.2.1 Combined AF4-ICP-MS

After careful evaluation of a range of analyse methods, development work was started on a method which combined a particle size separation step (Asymmetric Flow Field Flow Fractionation (AF4) and a particle detection/quantification step (Induction Coupled Plasma-Mass Spectrometry (ICP-MS)). For reasons of cost, complexity and expected availability in control laboratories it was decided not include on-line size measurements as part of the proposed method but rather to initially rely on a calibration of size and retention time. When combined together the two methods can be used to derive a particle number-size distribution by the following four steps.

- Firstly, the relationship between retention time and particle size would be determined for AF4 system using a range of mono-dispersed standards.
- Unknown samples would then be fractionated using the AF4 with simultaneous on-line determination of particulate mass concentration by ICP-MS
- The resulting fractograms of mass concentration and retention time would be converted into a mass-size fractogram by applying the calibration curve previously determined using size standards.
- Particle number size distribution would then be calculated from the experimentally determined mass-size distributions assuming that the particles have a known uniform chemical composition and that they have a known size/shape.

It is the view of the authors that the proposed combined method, AF4-ICP-MS, cannot satisfy all the needs of the definition but given the urgent need of the legislative bodies to have workable solutions to the analytical challenges posed by the nanomaterial definition it may, with suitable development, offer an interim solution. It is certainly true that electron-microscopy may give a more complete and reliable assessment of a materials nano-status this solution requires equipment and highly skilled operators which may be prohibitively expensive for routine use. While it is valid to say that the proposed AF4-ICP-MS method is not inexpensive if the whole system needs to be purchased, this effect is greatly reduced when it is considered that the most expensive component- the ICP-MS and its related infrastructure and technical support- is commonly available in the type of control laboratories who would be the principle user the type of protocol being examined in this

study. In this case the cost of upgrading the ICP-MS with a basic AF4 system is achievable at less than 15% the purchase cost of STEM and less than 10% of a TEM. This combined with the low running cost and ease of operation make it an interesting option to resolving this complex analytical problem.

2.3 Choice of particles and dispersion matrix for method development

The choice of nanoparticle/dispersant combination in this work was the fruit of a consideration of a number of relevant technical points as well as priorities from the most immediate needs of legislators. The main technical points were the following.

- The particles should be relevant to the current of future needs of the legislators
- The particle types must be specifically detectable by a ICP-MS
- It should be possible to obtain or fabricate suitable sample solutions for use in method development and in later validation studies.
- If possible, the detection (not quantification) should be possible with alternative methods such as UV or fluorescence spectroscopy to reduce the dependency on the more complex ICP-MS during method development.

From discussions with DG-SANCO, DG-ENT, DG-ENV a priority list of materials was produce as shown in Table 2-1

Table 2-1 Priority materials with possible nanomaterial content

Material	Priority	Specific applications
Ag	1	Food contact materials and additives
Titanium dioxide	1	Food contact materials and additives
Basic methacrylate copolymer	1	Nutritional supplements
Nanoclays	2	Food contact materials
Silicon dioxide	2	
Magnesium oxide	2	
Titanium nitride	2	
Carbon black	2	
Metal oxides	2	

From this list of nano-materials consideration was given to the higher priority materials to assess which had the greatest possibility of being developed into a working method in the minimum possible time. The basic methacrylate polymer was excluded as there is currently no sensitive method of specifically detecting and quantifying organic based nanoparticles. The nano-clay materials were excluded as these have variable non-standard compositions and contain many

elements which are ubiquitous in many possible test matrixes. The remaining two materials TiO_2 and Ag both offer advantage and disadvantages in terms of detectability and availability. After due consideration the choice of Ag was made for a number of technical reasons.

Firstly, Ag nano-particles are available commercially in highly monodisperse form and therefore adapted to the preparation of mixtures with particle number distributions which can be controlled and used for calibration. Secondly, Ag nanoparticles are, for a relatively wide range of sizes, plasmon resonant materials which mean that they exhibit material specific absorption in the UV-Visible range. This absorption is, for much of the particle size range, very intense and so permits the use of low-cost high sensitivity UV-Visible HPLC type detector for use in the method development. Unfortunately the intensity and peak wavelength of this absorption varies with the size (and shape) of the Ag particles and consequently this detector cannot reliably be directly used for routine quantification. However for the purposes of the method development required in this study the high sensitivity of the UV-Visible HPLC detector proved to be a very valuable tool and for much of the work this low cost method of detection was able to substitute than the much more complex and costly ICP-MS.

Finally, in step 2 of the general method it was intended to couple the output from the AF4 system to the ICP-MS for the real-time quantification of the particle mass in the eluted solution. In this case it is necessary that the particles can be efficiently and rapidly ionized in the plasma torch before sampling with the mass spectrometer. This can be achieved much more easily and reliably with relatively volatile metals particle such as silver as compared to highly stable refractory oxide such as TiO_2 thus reducing/eliminating the need for pre-digestion before introduction to an ICP-MS system. A further consideration was that digestion of TiO_2 generally requires the use of hydrofluoric acid which is not compatible with on-line work and represents a very serious safety hazard which should only be used in specially equipped laboratories with appropriately trained personnel.

Having decided that the nanoparticle most appropriate to this preliminary study would be Ag consideration was given to the type of liquid matrix in which to disperse the nanoparticles. As previously mentioned, at the time of writing this document there are no validated method nor reference standards for calibrations according to the EU recommended definitions. Consequently, for this study it was decided to utilise a very simple nanoparticle mixture which would eliminate the complications of pre-analysis sample preparation steps so allowing the laboratories to concentrate on the instrumental aspects of the process.

For reasons of technical simplicity and commercial availability it was decided that the study would utilise silver nanoparticles stabilised by sodium citrate-this being one of the most common form of

colloidal silver available commercially and therefore both relevant to future legislation and relatively easily sourced commercially.

2.4 Method developed for the separation of the particles

In summary, in this activity a method which combines a particle size separation (Asymmetric Flow Field Flow Fractionation (AF4)) step and a particle detection/quantification step (Induction Coupled Plasma-Mass Spectrometry (ICP-MS)) was developed and optimised for the analysis of aqueous dispersed silver nanoparticles. The method was developed specifically using sodium citrate stabilised silver nanoparticles which is one of the most common forms of this type of nanomaterial. Following an internal validation, the method has been documented in the form of a Standard Operating Procedure (SOP) designed to provide all the necessary information to allow it be applied by suitably equipped external laboratories. The specific details of the method can be found in the SOP which has been included as Annex 5 of this report.

3 Description of Study

The main objective of this phase of the ring-trial was to evaluate whether the basic particle separation methodology could be transferred from the development laboratory (JRC) to the other participant laboratories in the trial. One of the technical difficulties was that the different participant laboratories were equipped with a variety of different instruments (AF4 and ICP-MS) which meant that it was not possible to provide a single SOP with detailed instrument specific parameters. Instead, it was necessary to provide the participants with a generic procedure to optimize their own instrumental system starting from a basic set of recommended separation conditions. These conditions could then be modified until a predetermined minimum level of particle separation was verified based on numerical criteria specified in the SOP. Once the participants had verified that this phase had been completed successfully they could then apply this methodology to the analysis of two samples (A and B) whose composition (nanoparticle size and concentration) was not known to the participants.

3.1 Samples for calibration and method development

Nanoparticle dispersions containing near mono-dispersed silver nanoparticles were provided together with detailed information about the concentration and sizes of each. The nano-particle dispersions which were supplied had sizes in the range 10 to 100nm in size steps of approximately 10nm. Full details of the materials supplied will be given later in Section 5.

3.2 Standard Operating Procedure

The SOP, which is reported in Annex 5, provided the ring trial participants with the following:

i) A description of the nanoparticle standards

ii) A step-by-step description of particles storage and handling conditions along with instruction on how to optimize the separation procedure.

iii) A step-by-step description of the verification tests which had to be performed using predefined mixtures of two or more of the monodispersed nanoparticle solutions. From the data derived from these tests numerical values could be calculated to quantify the resolution and separation obtainable in each participant laboratory. Participants were requested to undertake method optimisation until predefined minimum values of resolution and separation could be achieved.

iv) Detailed instructions of how to prepare and analyse the two blind test samples (A and B) using the optimised separation conditions developed and verified in steps (ii) and (iii) above.

3.3 Test samples (A and B) with undeclared composition

The participants were supplied with two samples (A and B) which were declared to contain a mixture of two or more sizes of monodispersed nanoparticles whose identity (size and concentration) was not known to the participants. The preparation of these samples is detailed later in section 8.1 but basically the solutions were made by mixing accurately known volumes (determined gravimetrically) of the 50nm and 100nm particle dispersions to give a theoretical number ratio of 1:1 of the two sizes. In this way the mixture was designed to simulate a particle mixture which would be a borderline case under the terms of the EU recommended definition. To ensure uniformity across the test both samples A and B were prepared as two individual batches which were then aliquoted to make the samples for distribution in the trial. Sample A was provided at a high concentration of silver and was intended to be used by the participants after appropriate dilution in their laboratories. Sample B was provided at the correct working concentration and was designed to be used as supplied. In practice the two samples should have had exactly the same ratio of 50 and 100nm particles since the sample B was prepared in JRC laboratories by diluting a portion of the sample A. The samples were prepared in this way to permit the following

1) Evaluate the ability of the method to analyse samples close to the 50% number limit for the definition
2) Evaluate the repeatability/reproducibility by providing two samples which had the same identical ratio of 50 and 100nm particles
3) To provide data for use in determining appropriate sample concentrations for use in possible future trials.

It is well known that silver nanoparticle may be degraded by oxidation and exposure to light and that this effect is likely to be greater in highly dilute samples. By comparing the elution curves of sample B as supplied and sample A in a diluted state, information about the relative stability of the diluted sample be determined when exposed to full ring-trial conditions. It should be noted that prior to initiating the trial, stability tests on concentrated and diluted mixtures of nanoparticle silver were run by JRC and it was found that, under ideal storage condition there was minimal (<5%) loss of nanomaterial over the 4 week time period allowed for completion of the testing in the trial. This verification of sample stability could not take into consideration the addition deterioration which could occur during transport or non-ideal storage and handling conditions.

4 Description of Participants

From the response to the call to participate in this ring trial a total of eight national, regional or university laboratories were identified as having suitable facilities to participate, partially or fully, in the first stage of this study. Amongst the eight external laboratories, only four were equipped with AF4 and on-line ICP-MS while the remaining laboratories were limited to using UV spectrometry as the only on-line detection method. In addition, one of these four laboratories (no.7) had access to off-line ICP-MS and was able to analyse time resolved fractions of the AF4 elution.

Given the limited number of fully equipped laboratories in the trial, JRC also participated by analysing examples of the same batches of unknown samples A and B at a time point 3 weeks after the official start of the trail. This was done to increase the effective number of participants and to verify that the unknown samples could give representative results at a time point close to the maximum time allocated for completion of the study.

For the purposes of this report the results will be presented in an anonymous way with the external measuring laboratories being referred to by a code number (2-9) while JRC will be indicated as participant (1). Each external laboratory will be informed of their own identifier number.

4.1 Previous experience with AF4, ICP-MS and combined AF4-ICPMS

In the original call to take part in the ring trial all potential participants were asked to provide an indication of their previous experience in the fields of AF4, ICP-MS and combined AF4-ICP-MS. The information supplied is shown below in the Table 4-1

Table 4-1 Experience of participant laboratories

Laboratory No.	AF4 Experience	ICP-MS Experience	AF4-ICPMS	Participation (Full/Partial)
1	Y	Y	Y	Full
2	Y	Y	Y	Full
3	Y	Y	Y	Full
4	Y	Y	Limited	Full
5	Y	N/A	N	Partial
6	Y	N/A	N	Partial
7	Y	Y	Off-line	Partial
8	Limited	Y	Limited	Partial
9	Y	Y	Y	Full

As can be seen in Table 4-1 the majority of the laboratories have defined themselves as having an established experience in the use of AF4 while only 5, including JRC, have defined themselves as

having the necessary instrumentation as well as experience in the use of the full combination of AF4-ICPMS.

4.2 Equipment used by each participant

Given the highly specialized nature of this study it was foreseen that it would be difficult to guarantee that all participating laboratories could have access to the same type of instrumentation as was used to develop the method at JRC. Consequently, the SOP was prepared in a relatively generic manner which provided the necessary guidance to develop, optimise and verify analysis procedures for the various instrument combinations. The list in Table 4-2 shows the equipment used in the various laboratories.

Table 4-2 AF4 and ICPMS equipment available in each laboratory

Laboratory No.	AF4	ICP-MS
1	Postnova AF2000	Agilent ,7700x
2	Wyatt Eclipse 3+	Agilent ,7700x
3	Wyatt, Eclipse 3	Agilent, 7500 ce
4	Postnova AF2000 MF	PerkinElmer, Elan DRCII,
5	Postnova AF2000 MT	N/A
6	Postnova AF2000 MT	N/A
7	Postnova Analytics AF2000	Agilent 7500 (Off-line)
8	Wyatt, Dualtec	Thermo X Series 2
9	Wyatt, Eclipse 3+	Agilent, 7700x

As can be seen in Table 4-2 only five laboratories, including JRC, had access to AF4 with on-line analysis by ICP-MS. It should be noted that participant No.7 also had access to ICP-MS off-line and has supplied information on the results obtained using this intermediate solution. These results could not be directly integrated into the statistical analysis of the online studies but instead will be presented separately in Annex 4 of this document.

5 Description of materials used in test

The nanoparticle dispersions used in the trial were sourced from two commercial suppliers. The first supplier (A) was specialized in the production of high precision(size) gold and silver nanoparticles and was able to produce a series of nine nanoparticle dispersions with nominal sizes of 20,30,40…100 nm respectively. Each material was provided with a detailed technical specification by the manufacturer in which size and concentration were quoted. One additional material with a nominal size of 10 nm was purchased from a second supplier (B). This product was not accompanied

with a detailed technical specification but as this material was not for use in size calibration this was not a critical issue. Instead, this nanoparticle dispersion served only to verify that the AF4 elution method could adequately separate particles of 10nm from the void peak. Analysis of this material using CLS and Electron Microscopy (EM) showed that the size declared by the manufacturer was comparable with that effectively observed experimentally. To ensure that the data from the manufacturers A and B were reliable JRC undertook a more detailed series of independent analysis to verify the size, mono-dispersivity and concentration of the particles.

5.1.1 Size and monodispersivity of silver nanoparticle solutions

The particles used in the study were used as size calibrants with the size measurements being given by the values provided in the manufacturer certification. The sizes, along with other details, are reported in Table 5-1.

Table 5-1 Size and concentration values for each type of Ag nanoparticle distributed in the study

Standard	Diameter TEM [nm]*	First Standard Deviation[nm]*	Hydrodynamic Diameter(DLS)[nm] *	Concentration by ICP-MS[µg mL^{-1}]**
AgNP10	10(±4)***	N/A	N/A	17.7
AgNP20	19.6	1.6	N/A	19.1
AgNP30	32.3	3.2	44.8	13.2
AgNP40	40.6	3.0	53.7	18.3
AgNP50	52.4	5.9	58.1	19.8
AgNP60	57.4	4.0	66.7	19.4
AgNP70	68.5	4.2	69.0	21.1
AgNP80	77.1	6.4	83.0	20.0
AgNP90	88.9	4.3	86.8	18.1
AgNP100	99.4	7.0	97.7	18.7

* Value from manufacturer specification certificate unless state otherwise

** Value measured in JRC laboratories

*** Nominal non-certified value by manufacturer

5.1.2 Verification of mono-dispersivity by Centrifugal Liquid Sedimentation (CLS)

To ensure that the particles used for method development were fit-for-purpose it was necessary to verify that the expected degree of mono-dispersivity was respected. To do this each type of particle was analysed by CLS to check the size distribution of each materials and to verify the presence of any eventual aggregation. The results of these analyses are shown below in Figure 3. The results confirm that the particle distribution are narrow mono-modal for all sizes except the those of 90nm and

100nm each of which shows a small secondary peak at higher size. This is consistent with the presence of a low concentration of dimers.

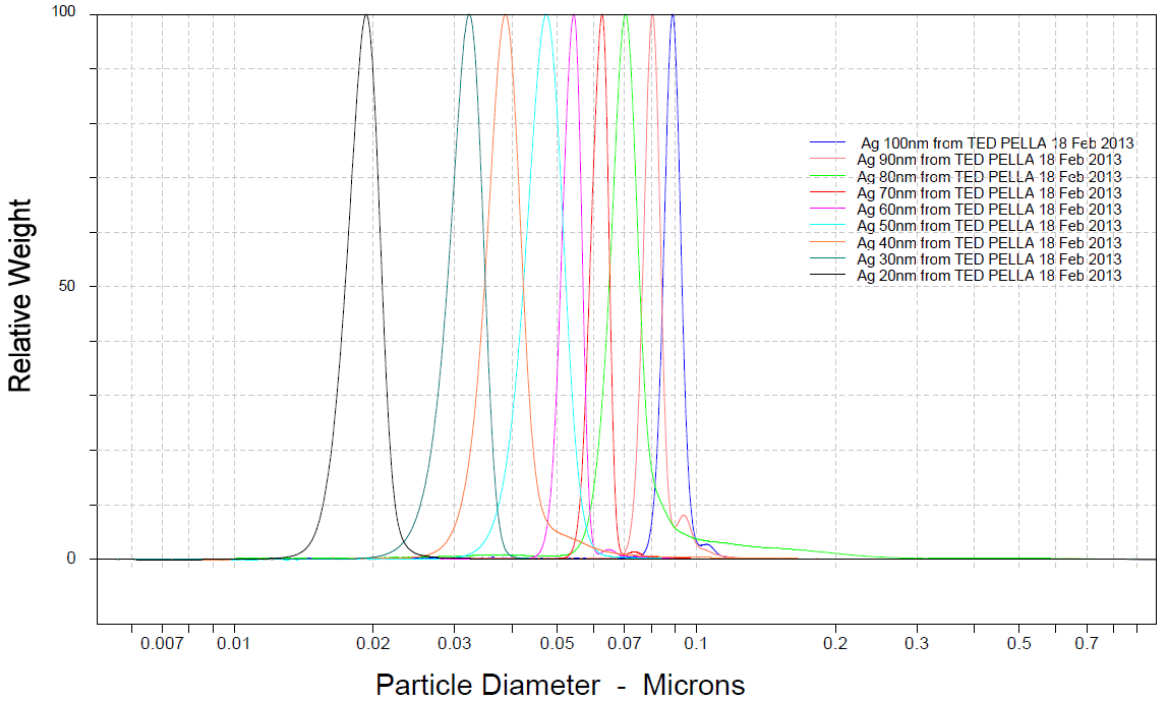

Figure 3 CLS analysis of pseudo standard solution of Ag nanoparticles

5.1.3 Verification of temporal stability under controlled conditions

During this study samples were required to be stored at 4°C and once opened it was recommended that they be reclosed under an inert atmosphere of N_2 or Ar. It was further requested that the samples be utilised within 4 weeks of delivery. These conditions were established following the results of a preliminary stability study in which nanoparticle mixtures were prepared and periodically analysed using CLS during a period of up to 4 weeks. Examples of these results are presented in Figure 4 where it can be seen that the particle mixtures show only minimal variation in quantity and size distribution during this time period.

In addition to this, representative samples of the calibration solutions and the unknown samples A and B were stored at JRC and periodically analysed using CLS (samples A and B) and UV-Visible Spectrometry (calibration solutions). By undertaking these analysis during the 4 week period following dispatch of the test samples it was possible to confirm (see sections 6.1 and 6.2) the temporal stability of the materials.

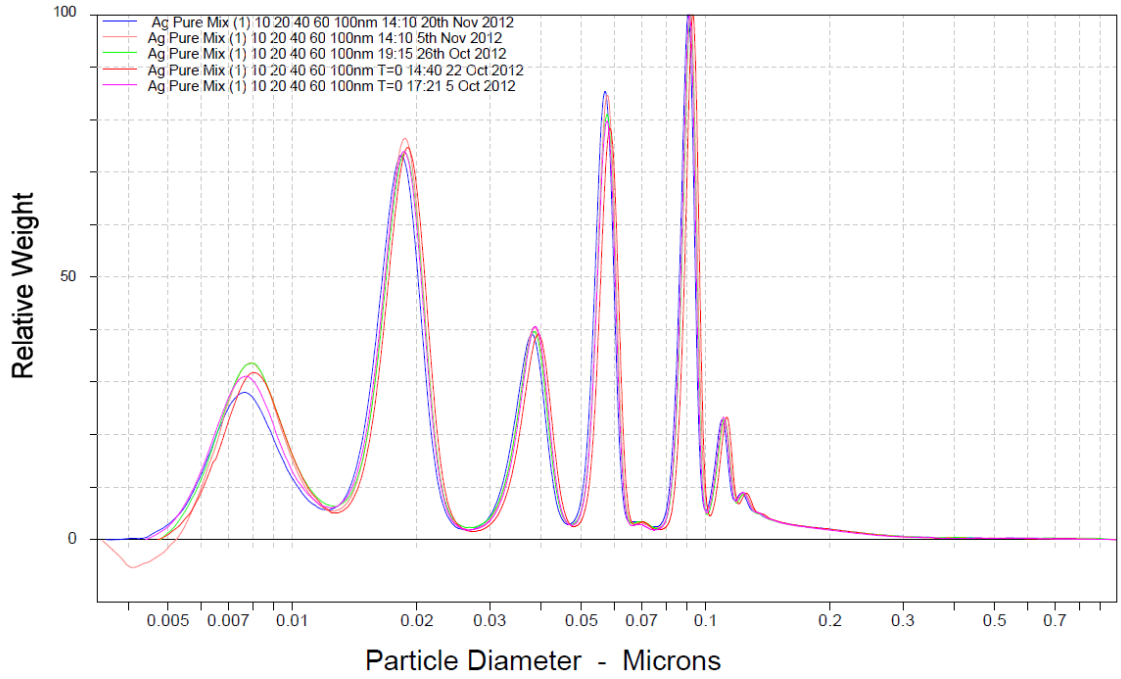

Figure 4 CLS measurement of undiluted development mix from Time=0 to 4 weeks

6 Results obtained during the Inter-Laboratory Comparison (ILC)

The experimental results collated in this study cover three main aspects of ILC.

- Verifying the stability of the test materials during the course of the ILC (JRC only)
- Evaluation of effective separation (JRC and all participants)
- Measurement of unknown samples A and B (JRC and all participants)

Each of these three aspects will be considered in separate sections with the current section being dedicated to the verification of the stability of the test materials used in the study. As has already been outlined in the Section 5.1.3 prior to initiating the organization of the ILC, JRC has confirmed the temporal stability of similar silver nanoparticle solutions and mixtures over time periods equal or greater than that consider necessary to complete the experimental phase of the ring-trial. However, given the relatively unstable nature of silver nanoparticles it was thought prudent to also repeat similar tests on the random examples of the materials distributed in the ILC. These tests were conducted periodically over the 4 week time period in which the participants were requested to complete their measurements. In this way it was possible to evaluate the extent that nanoparticle instability could have on the quality of results and to ensure that samples would remain suitable for use through-out the duration of the trial.

6.1 Verification of the stability of single pseudo-standards during test period

Stock solutions of mono-dispersed silver nanoparticle-dispersions were monitored to verify their stability over a time period of 21 days from the time when the trial materials were dispatched to the

participant laboratories. This was done by determining the area of the absorbance-peaks of each stock solution (AgNP10nm, AgNP20nm, AgNP30nm, AgNP40nm AgNP50nm, AgNP60nm AgNP70nm, AgNP80nm AgNP90nm, and AgNP100nm). Figure 5.1 shows an example of how peak areas were integrated. Measurements were conducted once a week for three weeks. Fresh aliquots from the same vial were used each time. Spectra integration-windows for each stock solution/particle dimension are reported in Table 6-1 and Table 6-2.

Except for AgNP10 and AgNP 90, the overall loss was below 1%.

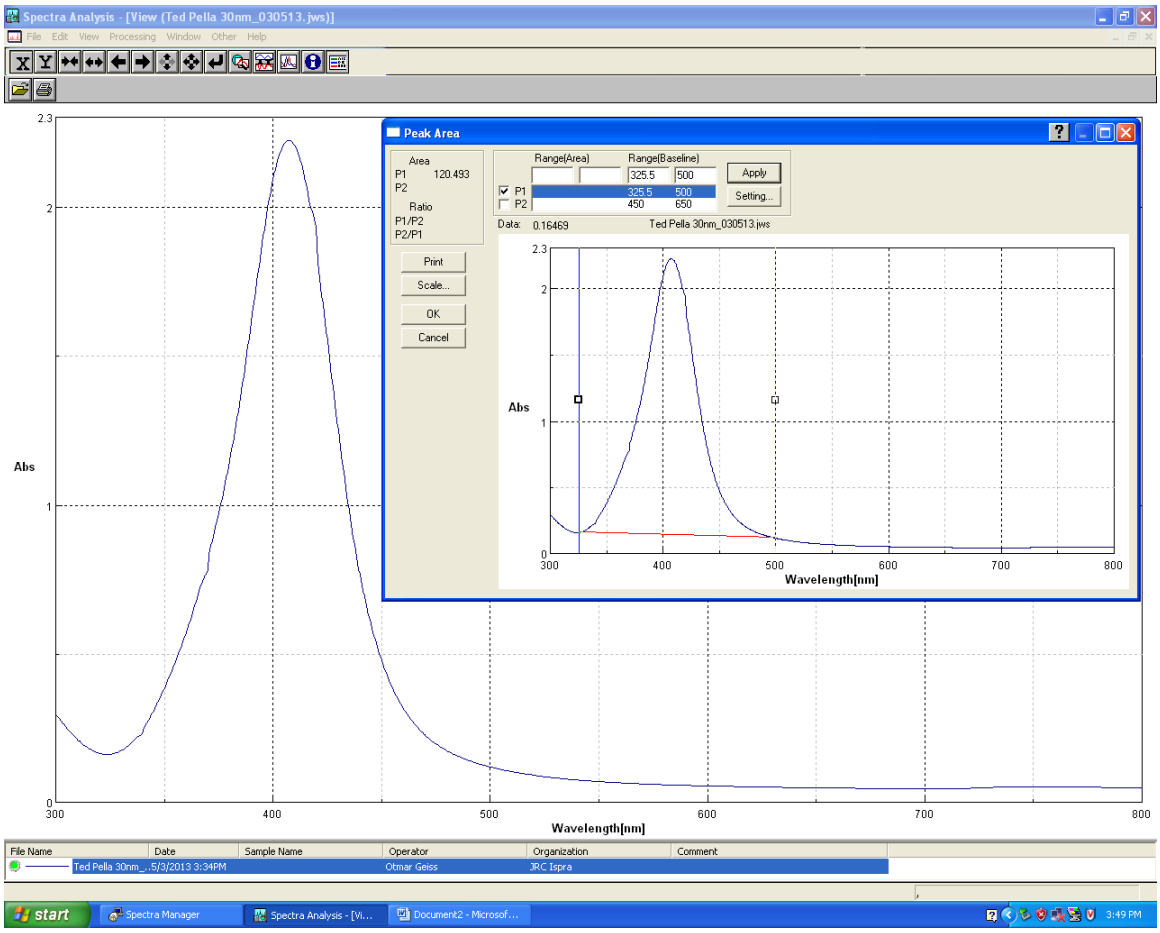

Figure 5 Example of integration window (here for AgNP30nm)

Table 6-1 Spectra window used for each stock solution

Stock Solution	Integrated Spectra Window [nm]
AgNP 10 nm	321.5 – 500
AgNP 20 nm	321.5 – 500
AgNP 30 nm	325.5 – 500.1
AgNP 40 nm	326.5 – 550.5
AgNP 50 nm	324 – 600
AgNP 60 nm	324 – 601.5
AgNP 70 nm	323 – 657
AgNP 80 nm	321.5 – 800
AgNP 90 nm	321.5 – 800
AgNP 100 nm	321.5 – 800

Table 6-2 Integrated absorption peak areas for each monodispersed stock suspension

Date	12 April 2013	19 April 2013	24 April 2013	3 May 2013	
Days elapsed	0	7	12	21	
Stock Solution	Integrated absorption peak area				Decrease of peak area on 21 days [%]
AgNP 10 nm	134.147	133.105	132.302	130.087	3.0
AgNP 20 nm	166.842	166.269	166.029	165.404	0.9
AgNP 30 nm	121.064	120.72	120.774	120.493	0.5
AgNP 40 nm	171.613	171.45	171.489	171.169	0.3
AgNP 50 nm	181.015	181.039	181.307	180.838	0.1
AgNP 60 nm	175.314	175.267	175.32	175.101	0.1
AgNP 70 nm	198.068	197.662	197.53	197.2	0.4
AgNP 80 nm	192.248	191.867	192.044	191.883	0.2
AgNP 90 nm	165.407	157.57	157.614	157.715	4.7
AgNP 100 nm	170.77	170.45	170.314	169.645	0.7

The total decrease in absorption in the given integration windows was <5% for all stock solutions.

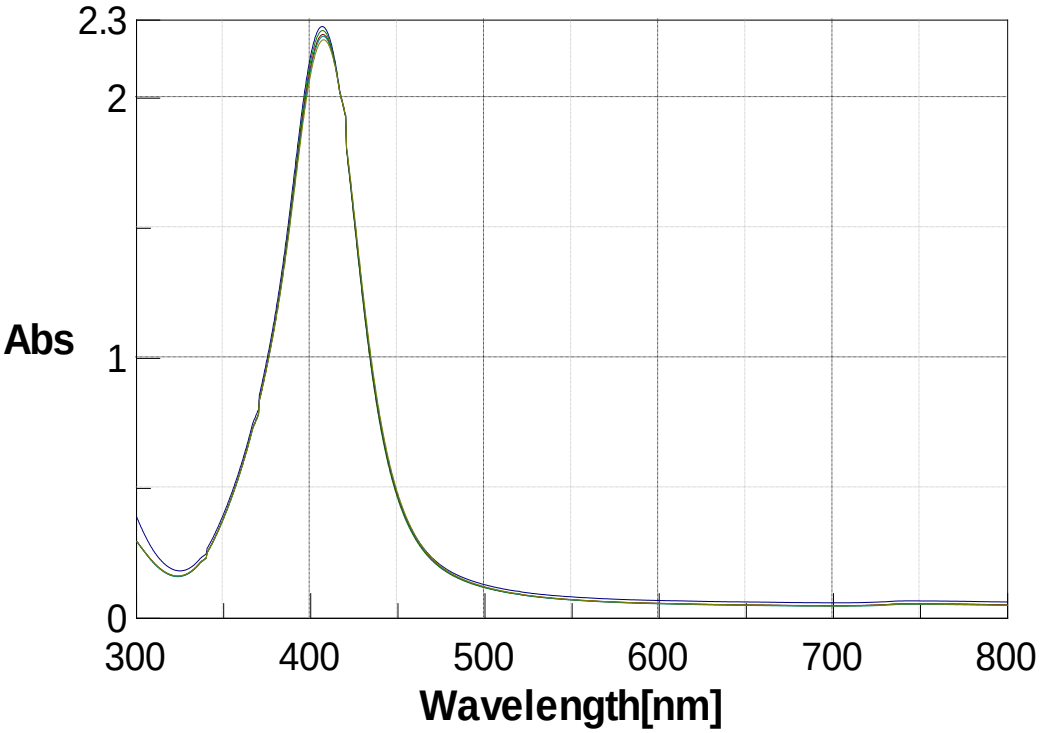

Figure 6 Overlaid absorption spectra for AgNP30nm during the testing period of the ring trial

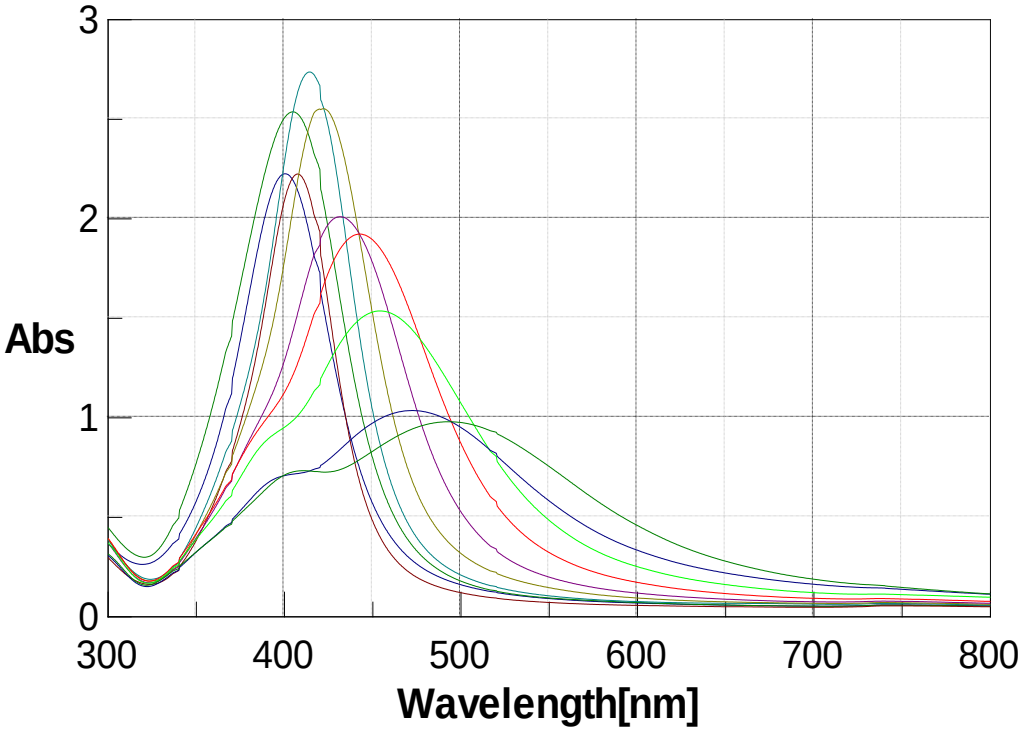

Figure 7 Spectra of all sizes of stock-solutions overlaid (3-May-2013)

6.2 Verification of the stability of unknown sample B during test period

In addition to this a series of analysis were conducted on Sample B to verify whether the diluted test material was stable over the time scale of the study. To do this, samples were analysed by CLS periodically during the allocated time period. The figure below (Figure 8) shows CLS data

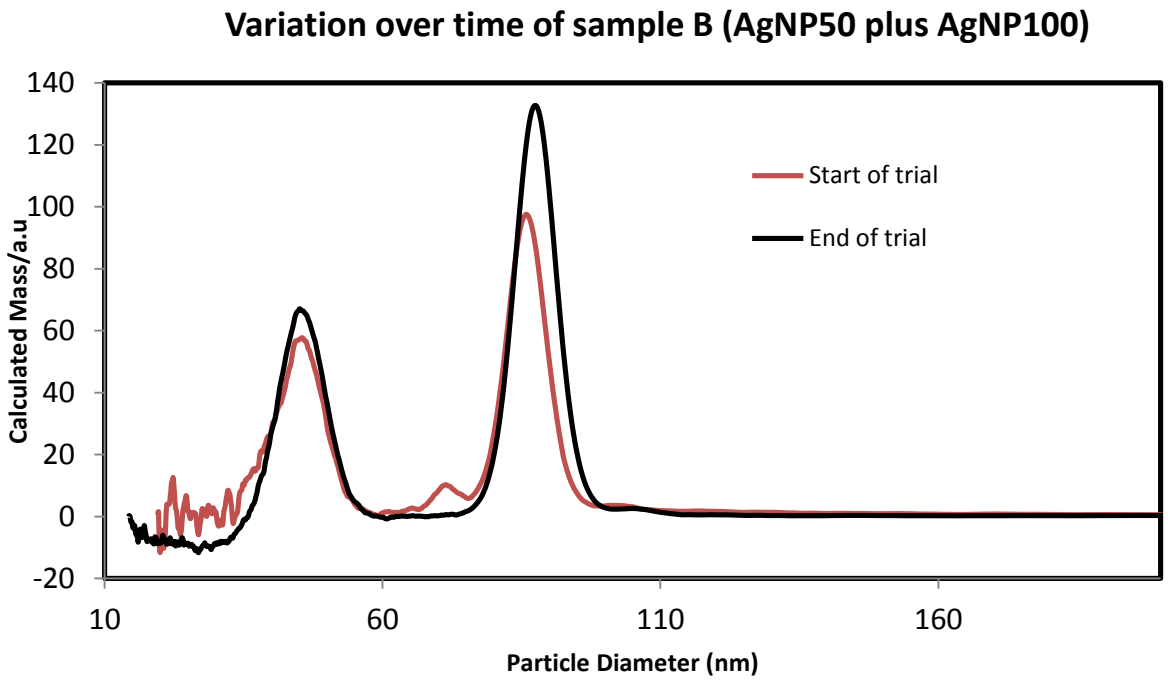

Figure 8 CLS analyses of sample mix B at start and end of recommended trial period

From these result it can be seen that although there was some limited variation in the relative quantities of the nominally 50 and 100nm materials but that there was no serious degradation with the material retaining a clear bimodal distribution.

7 Results obtained during method optimisation

As previous discussed the participants were provided with a series of silver nano-particle pseudo standards which were to be used by each participant to optimise and verify the separation process developed for their own specific combination of apparatus. This was to be done in 4 steps.

- Evaluation of effective separation from void peak
- Evaluation of 10/40nm peak separation by quantitative determination of resolution
- Evaluation of 40/100nm peak separation by quantitative determination of resolution
- Evaluation of 20/60nm peak separation by quantitative determination of resolution
- The following sections will illustrate the results obtained in each of these steps.

The analysis of the unknown samples A and B to determine size and concentrations will be fully detailed in the successive chapter where both the data and the statistical analysis will be presented.

7.1 Evaluation of effective separation from void peak

The first stage in the method optimisation was to verify that the peak of the 10 nm silver particles could be adequately separated from the void peak. To evaluate the effectiveness of this separation the capacity factor k was calculated according to the formula below

$$k'(AgNP10) = (tR(AgNP10) - tV(void\ peak)) / tV\ (void\ peak)$$

In this study an adequate level of separation was consider to be achieved when the value of k was greater than 0.6. The numerical results calculated by the participant laboratories were collated and can be seen in the Figure 9 which shows that all but 2 laboratories achieved a capacity factor which was at or above the required value of 0.6.

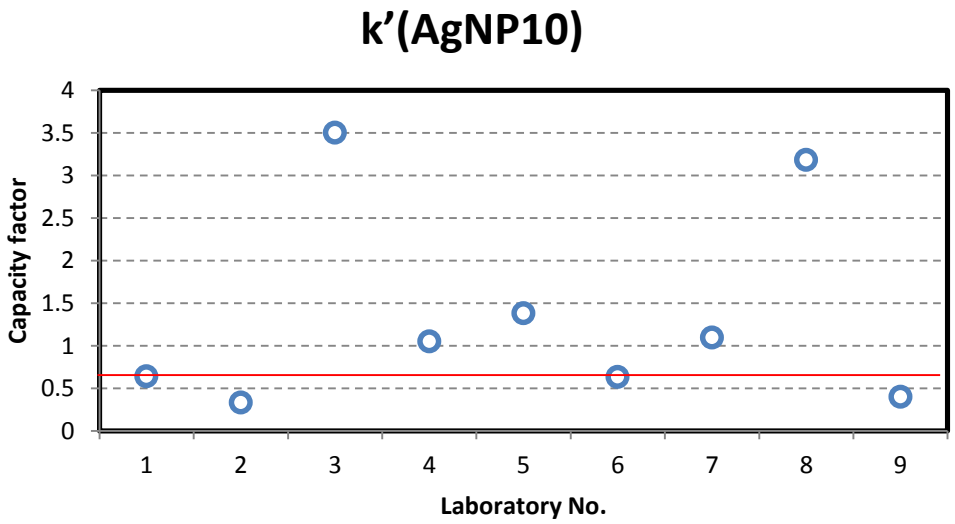

Figure 9 Reported capacity factor k for separation of void peak and nominal 10nm

7.2 Evaluation of 10/40nm peak separation by quantitative determination of resolution

The second stage of the optimization process required that the cross flow profile be adjusted to achieve certain minimum levels of peak separation when using sample mixtures of known particle size. To assess this it was requested that the degree of separation of mono-dispersed species be determined by calculating the peak *resolution* from experimental data. The resolution of two species, A and B, is defined as

$$R = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$

Where *W* is the peak width at the peak base and t_R is the retention time of peaks A and B. Baseline resolution is considered to have been achieved when the value of R is great than or equal to 1.5.

The first sample in the optimisation was the trimodal mixture containing particles with size 10nm, 40nm and 100nm. From the fractograms obtained using UV detection it was requested that the resolution be calculated for the separation of the 10 and 40 nm peaks and then the 40 and 100 nm peaks. The numerical results obtained are shown in Figure 10 and Figure 11.

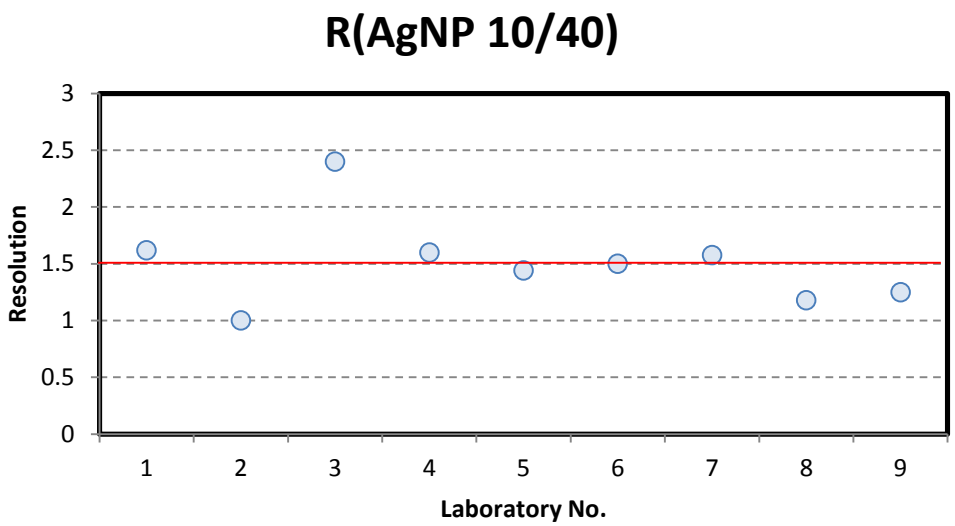

Figure 10 Evaluation of 40/100nm peak separation by quantitative determination of resolution

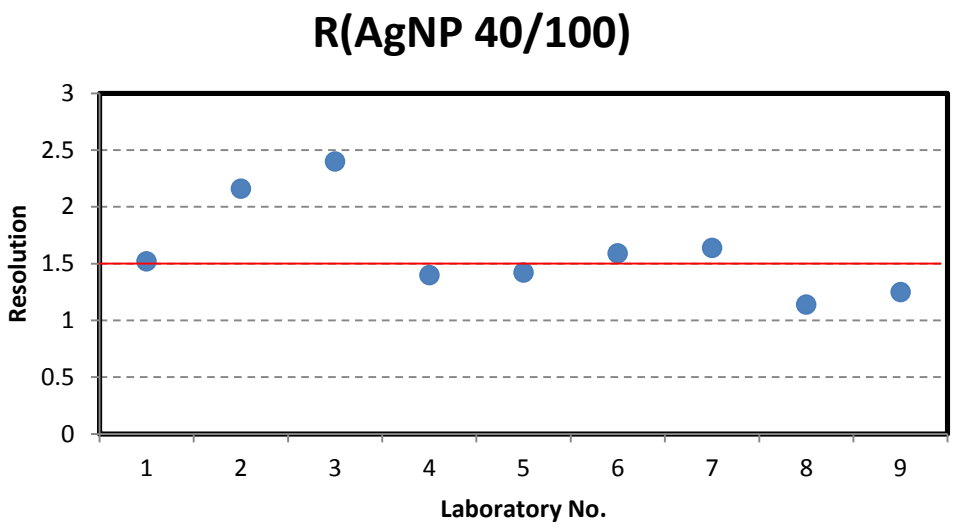

Figure 11 Evaluation of 40/100nm peak separation by quantitative determination of resolution

7.3 Evaluation of 20/60nm peak separation by quantitative determination of resolution

The second stage of this part of the evaluation used a bimodal mixture of 20 and 60nm. In this case only one laboratory was able to clearly improve on this value while six were either at or slightly above the required value.

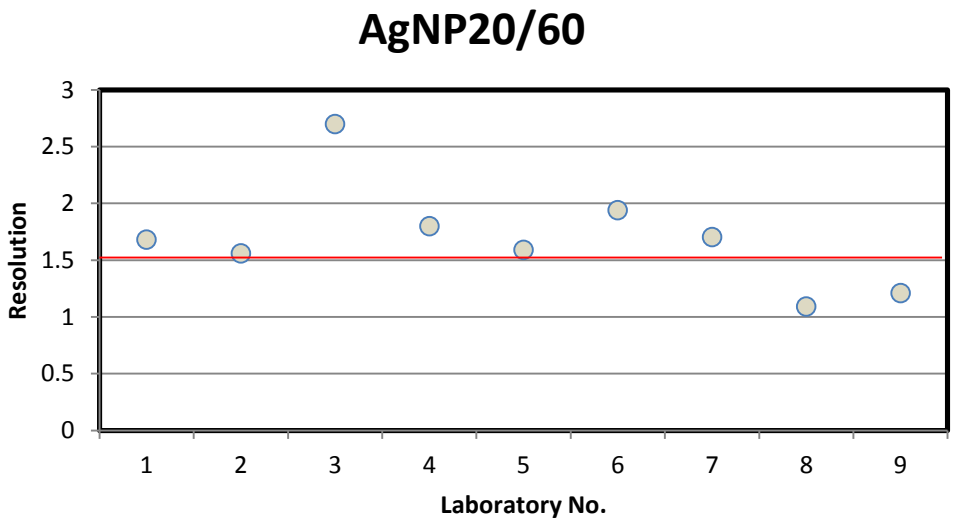

Figure 12 Evaluation of 20/60nm peak separation by quantitative determination of resolution

7.4 Qualitative evaluation of separation by measurement of resolution

The final evaluation of the peak resolutions was to analyse a mixture of 40nm and 60nm particles and assess whether peak separation could be achieved. From high resolution CLS measurements it has been seen that these two materials are only just baseline separable and therefore would be a

challenging sample with which to evaluate the true separating capabilities of each optimised AF4 system. It was not generally expected that it would be possible to achieve baseline separation with the AF4 systems but an examination of the resulting elution curves could be used to verify the relative quality of separations achieved in in the trial compared to that of JRC. In this consideration a qualitative ranking of the separation relative to laboratory 1 was made and is shown in Table 7-1. The original elution curves as supplied by the participants are reproduced in Annex 1.

Table 7-1 Qualitative assessment of AgNP40/60 separation

Laboratory	Separation achieved- relative to laboratory 1
1	+/-
2	-
3	++
4	+
5	+/-
6	+/-
7	++
8	+/-
9	-

7.5 Elution profiles resulting after the optimization process

Following the previously described stages of optimization and verification it was possible for each laboratory to establish for themselves a single set of experimental parameters which were to be used in the rest of the analyses undertaken in the course of the ILC. The main parameters used in the optimised separation procedures of each laboratory are listed in Table 7-2.

7.6 Time-Size calibrations obtained

Using the previously described elution profiles the participant laboratories were requested to generate calibrations curves to correlate particle elution time with size. The calibration curves obtained can be seen in Figure 13.

Table 7-2 Elution profiles used by each laboratory after optimization

Laboratory	Injection Flow [mL/min]	Injection/ Focusing Time [min]	Detector Flow [ml/min]	Initial Cross-Flow [mL/min]*	Elution profile
1	0.2	5	0.5	1	1 to 0.1 (linear decrease within 40min then constant)
2	0.2	5	0.5	0.5	Exp. decrease of cross flow from 0.5 ml/min to 0.1 ml/min
3	0.2	7	1	1	Linear 1 to 0.1ml/min in 30min
4	1.5	5		1.5	
5	0.2	3		2	
6	0.2	5		1.5	1.5- 0.1 linear decrease in 35 min then 10 min constant at 0.1)
7	0.2	5	0.5	2	
8	0.2	5	0.5	1	1 constant
9	0.2	3	0.5	1	1 to 0.1 (linear decrease within 40min then constant)

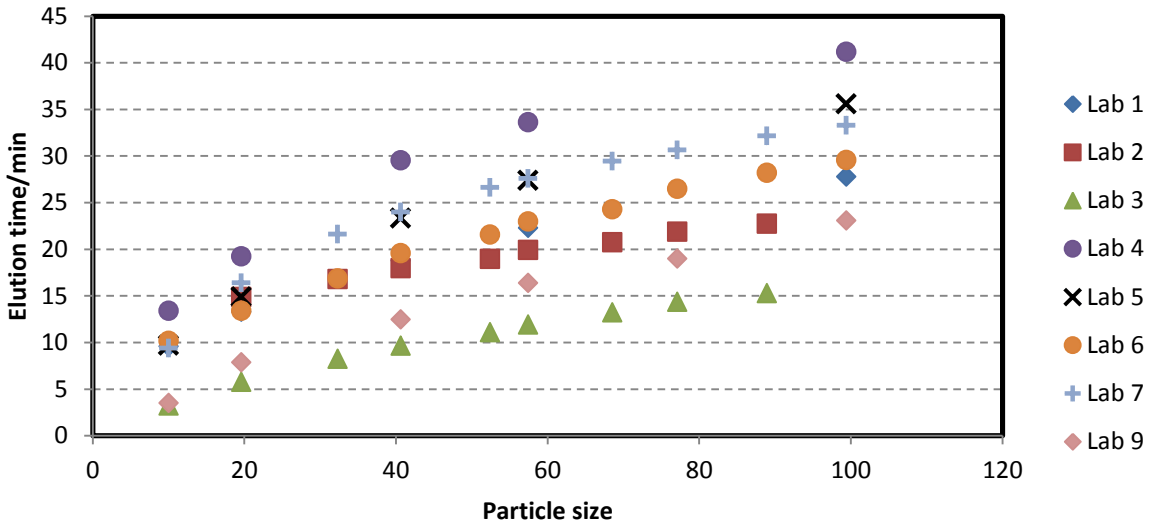

Figure 13 Calibration curves of size and elution time as reported by each laboratory

8 Analysis of unknown samples A and B

To evaluate the effectiveness of the methodology, participants were requested to analyse two bi-modal samples, each of which contained a mixture of two size(s) of silver nanoparticles in a concentration range between 100-1000 ng mL^{-1}. The samples were labelled **"Unknown Sample A",** and **"Unknown Sample B"** and no information was provided about particle sizes or concentrations. "Unknown Sample A" had to be diluted 1:20 with ultrapure water prior to analysis, whereas "Unknown Sample B" was ready to be analysed without any dilution.

8.1 Description of "Sample A" and "Sample B"

Unknown Samples A & B both contained two monodispersed particle sizes each[1]:

Size 1:	52.4 nm
Size 2:	99.4 nm

The concentrations of these two particle sizes were chosen in order to represent a borderline case with respect to the definition of a nanomaterial (50% of particle number <100nm and 50% of particle number > 100nm). Due to the non-availability of particles with a size >100nm, particles with 99.4nm of diameter were considered to correspond to >100nm as this does not considerably change calculations).

The diameters of the silver nanoparticles contained in the samples were to be calculated from the calibration curve previously prepared by plotting elution time against particle size. The concentration of the eluted silver fractions was to be determined with ICP-MS (either online or offline).

8.1.1 Preparation Unknown Sample A:

Exactly 21.35555g of the monodispersed AgNP(100nm) standard (c=18.7 µg mL^{-1}) and exactly 2.59138 g of the monodispersed AgNP(50nm) standard (c=19.8 µg mL^{-1}) were weighed into a 25 mL volumetric flask and brought to volume with ultrapure water.

Concentrations in sample A were 15.97395 µg mL^{-1} of Ag(100nm) and 2.052373 µg mL^{-1} of Ag(50nm) respectively. Aliquots of this solution were sent to trial participants labelled as "Unknown Sample A". Sample A was to be used after a dilution of 1:20 which results in the following final concentrations.

Sample A (diluted)	c(AgNP50nm)	= 103 µg L^{-1}
	c(AgNP100nm)	= 798 µg L^{-1}

8.1.2 Preparation of Unknown Sample B:

Exactly 2.48689 g of the undiluted "Unknown Sample A" solution were weighed into a 50 mL volumetric flask and brought to volume with ultrapure water. The resulting concentrations in sample B were therefore calculated to be the following

Sample B (as supplied	c(AgNP50nm)	= 102 µg L^{-1}
	c(AgNP100nm)	= 795 µg L^{-1}

8.2 Determination of sizes and concentrations in "Unknown Samples"

Participants of the current method performance exercise were asked to report both particle sizes and concentrations for "Unknown Sample A" and "Unknown Sample B". Based on these results, a statistical evaluation was conducted.

8.2.1 Reported values of concentration

The concentrations of silver reported by each exercise-participant are shown in Table 8-1. The ICP-MS data obtained by laboratory 7 are presented for completeness but have not been included in the statistical analysis which was performed only for the laboratories able to do the on-line analysis.

Table 8-1 Concentrations (µg L^{-1}) determined for "Unknown Sample A" and "Unknown Sample B"

	Unknown Sample A[2]		**Unknown Sample B**		
Laboratory Code	**AgNP (50 nm)**	**AgNP (100 nm)**	**AgNP (50 nm)**	**AgNP (100 nm)**	**Calibration Method**
Ref. values	103	798	102	795	N/A
1a	103.9	863.7	73.1	742	Pre-channel with particles
1b	76.1	631	58.3	575.6	Post channel with ionic silver
2	185.7	368.6	131.7	663.3	Post channel with ionic silver
3	106.9	398.6	109	544	Post channel with ionic silver
4	42.6	400.3	32	397	Post channel with ionic silver
5	72	680	63	750	Post channel with ionic silver
6	N/A	N/A	N/A	N/A	
7	14.3 11.7	243.9 125.2	--- 17.1	231.2 258.5	Off-line run 1 Off-line run 2
8*	N/A	N/A	N/A	N/A	
9	61.4	542.4	91	661	Post channel with ionic silver

*Note: For technical reasons laboratory 8 was not able to undertake the analysis of the same samples A and B at the required time and was supplied with similar but not identical samples at a later date: The results obtained were therefore not comparable and have not been included in the statistical evaluation.

[2] Concentrations of the 1:20 diluted solutions are reported for "Unknown Sample A"

8.2.2 Data distribution

Figure 14 to Figure 17 show the distribution of results obtained for the determination of concentrations of the two particle sizes in “Unknown Sample A“ and “Unknown Sample B“. The dark blue lines represent the smoothed distribution of all test results; the light blue lines represent the cumulative distribution.

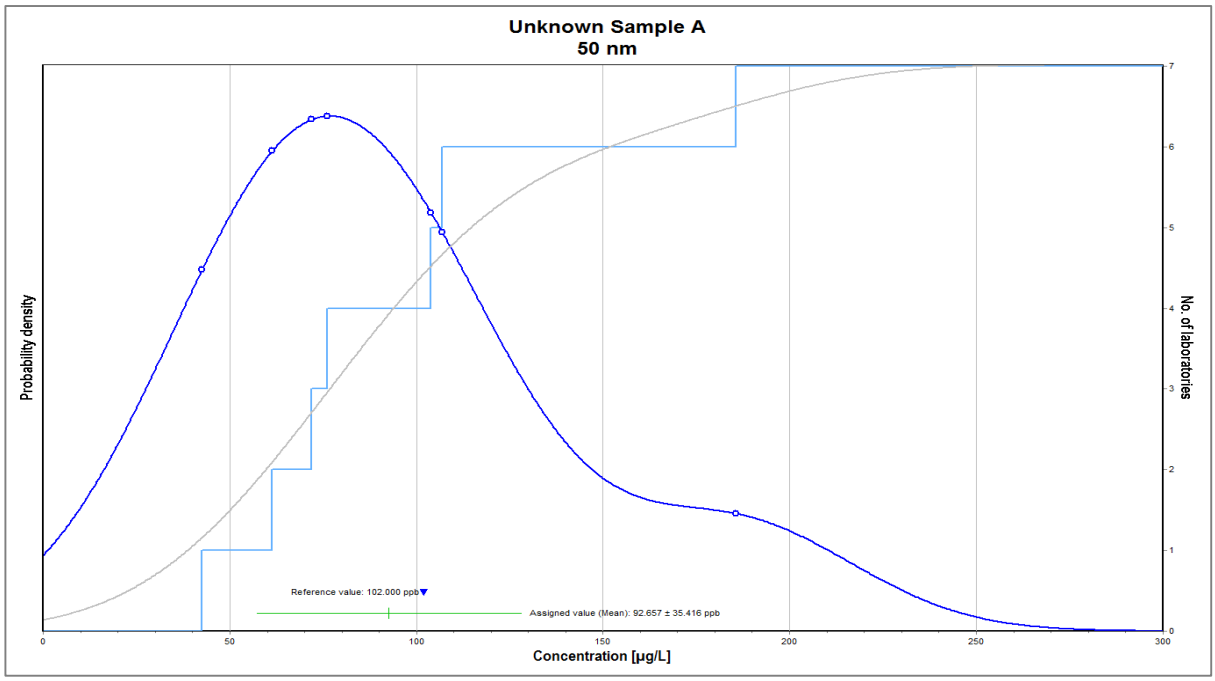

Figure 14 Data distribution for “Unknown Sample A”, 50nm

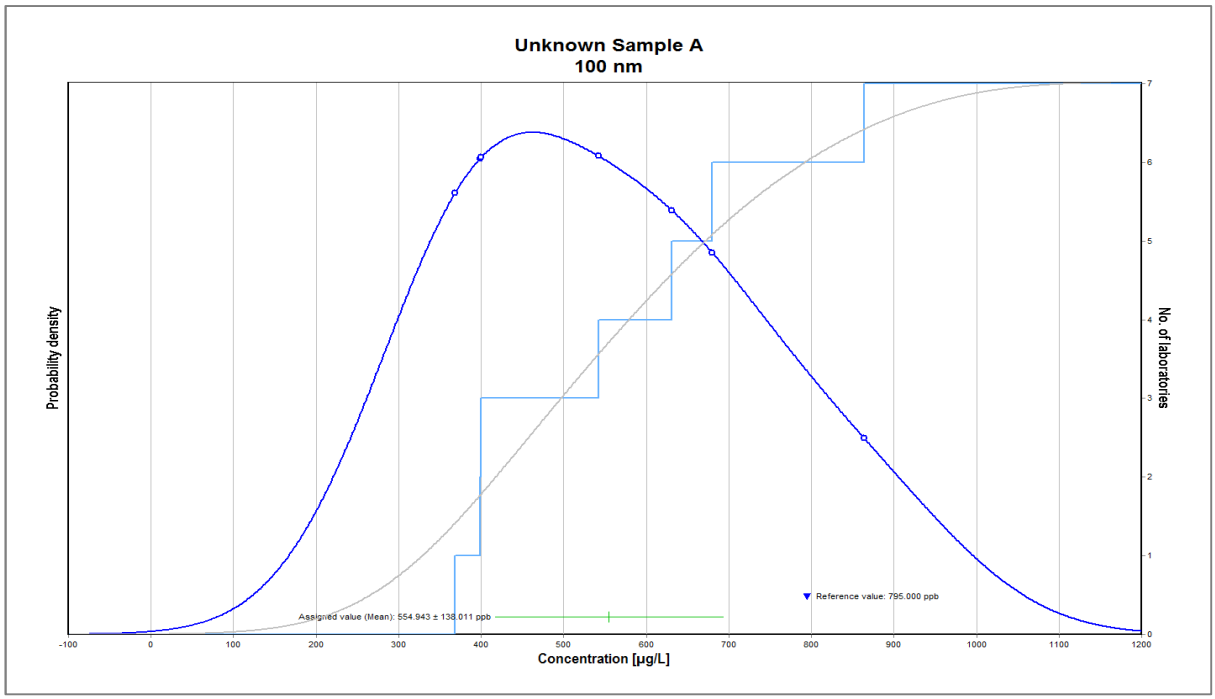

Figure 15 Data distribution for “Unknown Sample A”, 100nm

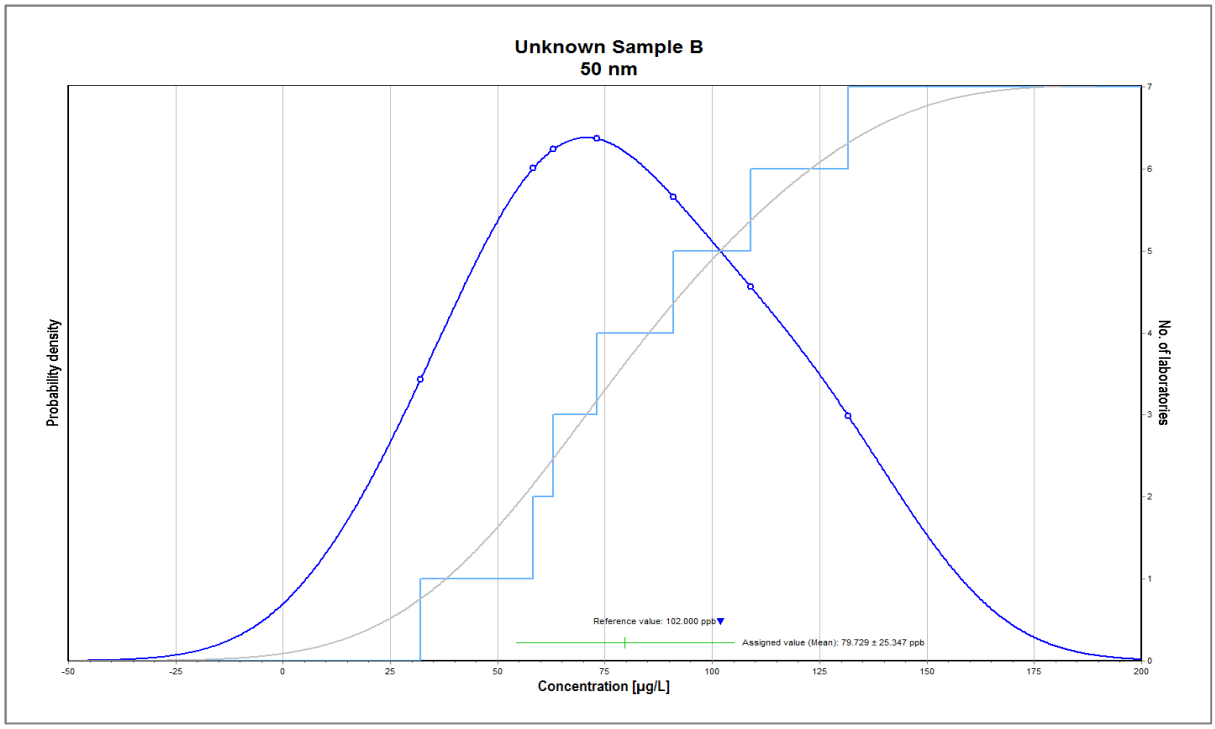

Figure 16 Data distribution for “Unknown Sample B”, 50nm

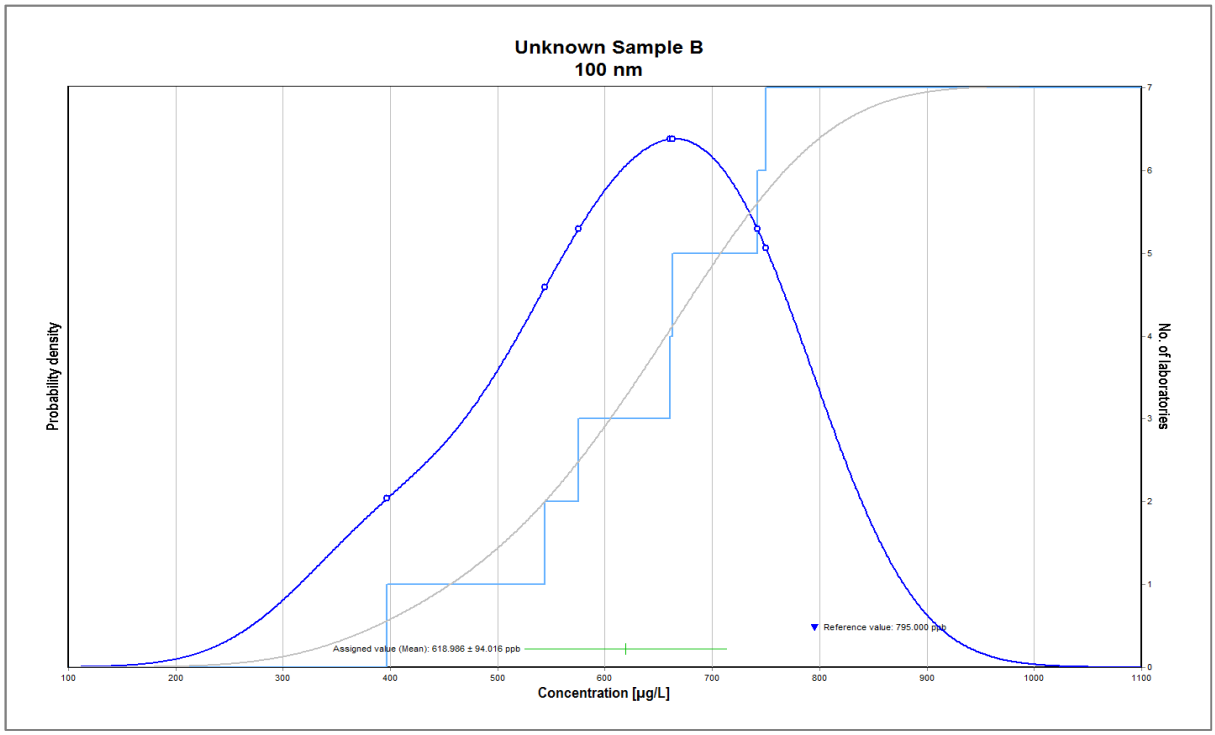

Figure 17 Data distribution for “Unknown Sample B”, 100nm

Although not perfectly symmetric, the data are sufficiently normally distributed to perform statistical analysis according to ISO 5725.

8.2.3 Outlier Tests

Data has been tested for outliers (Grubbs and Cochran). No outlier has been identified.

8.2.4 Statistical Parameters according to ISO 5725-2*

Results for some of the principal statistical parameters are shown in Table 8-2

Table 8-2 Results of selected statistical parameters

	Unknown Sample A		Unknown Sample B	
	AgNP(50 nm)	**AgNP(100 nm)**	**AgNP(50 nm)**	**AgNP(100 nm)**
Unit	µg mL^{-1}	µg mL^{-1}	µg mL^{-1}	µg mL^{-1}
No. of labs that submitted results	7	7	7	7
Mean	92.6	554.9	79.7	618.9
Reference Value	103	798	102	795
s.d.	46.8	182.5	33.5	124.3
Rel. s.d.	50.6	33.0	42.1	20.1
Limit of reproducibility, R(2.80 x sR)	131.181	511.199	93.886	348.239
Standard Error	17.708	69.005	12.673	47.008
Lower confidence limit of LPOD	57.241	416.932	54.382	524.970
Upper confidence limit of LPOD	128.073	692.954	105.075	713.001
Outliers	-	-	-	-

*ISO 5725-2:1994 Accuracy (trueness and precision) of measurement methods and results -- Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method

The following charts (Figure 18 to Figure 21) represent the raw data, plotting laboratory-identifications on the x-axis and determined concentrations on the y-axis. Values are plotted in ascending order. The blue dotted line represents the mean concentration, the green lines the standard deviations and the red line the theoretical reference value (the “real” value).

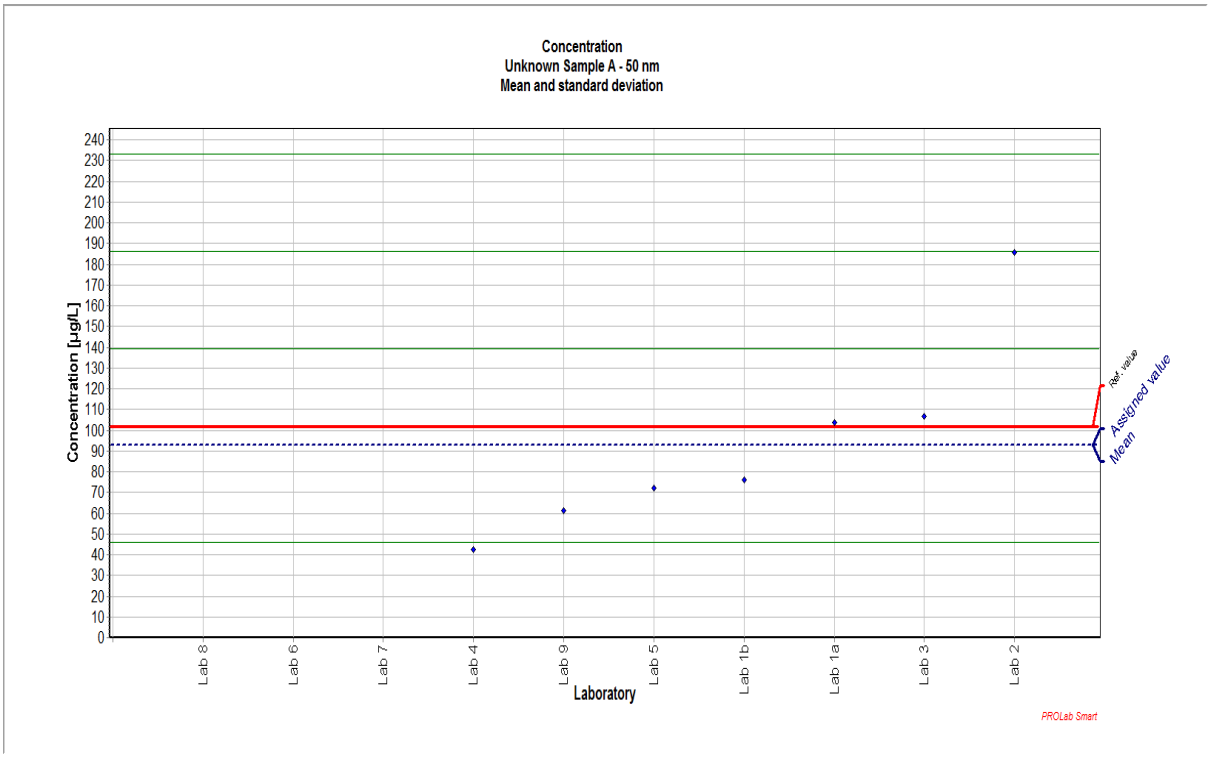

Figure 18 Unknown Sample A – AgNP(50nm)

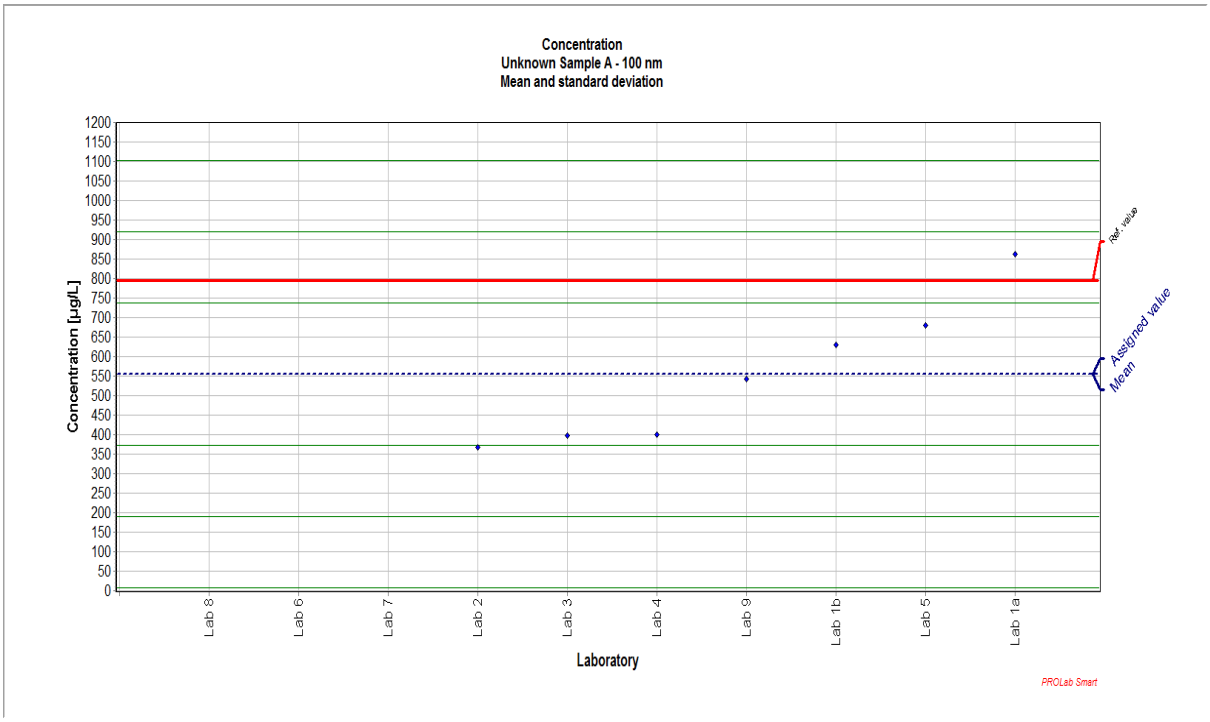

Figure 19 Unknown Sample A – AgNP(100nm)

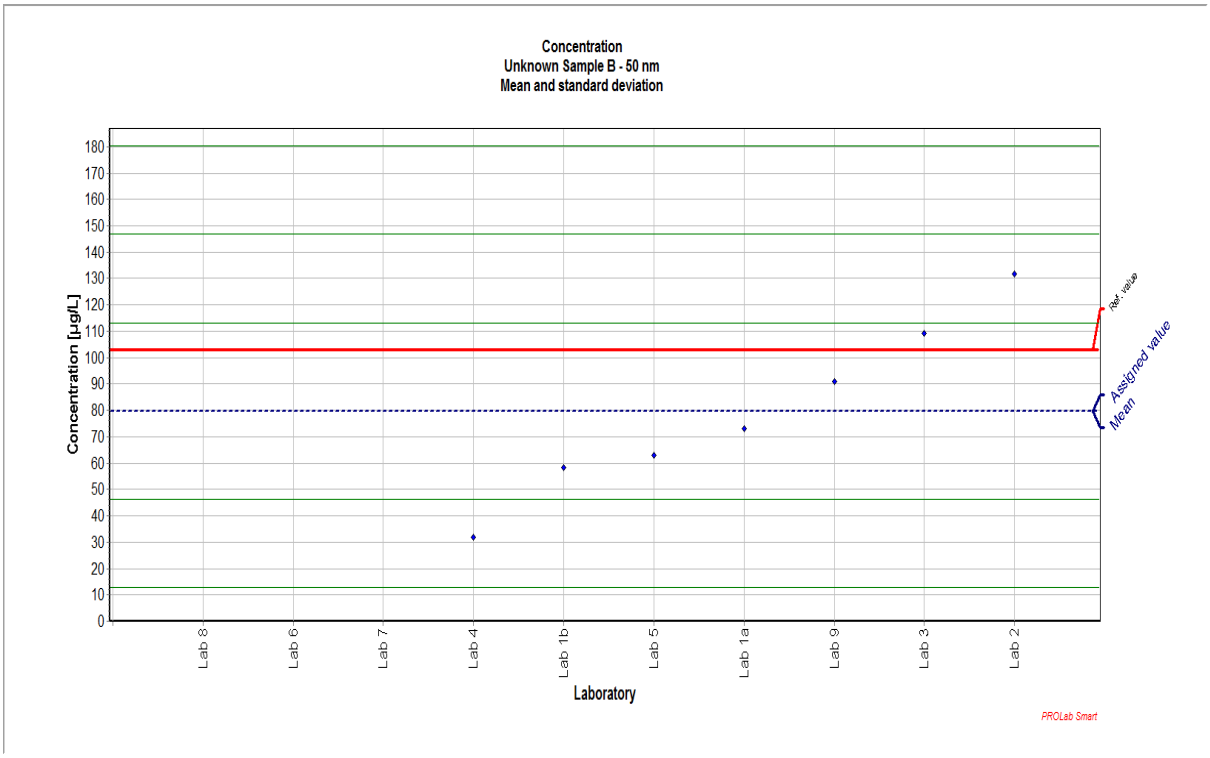

Figure 20 Unknown Sample B – AgNP(50nm)

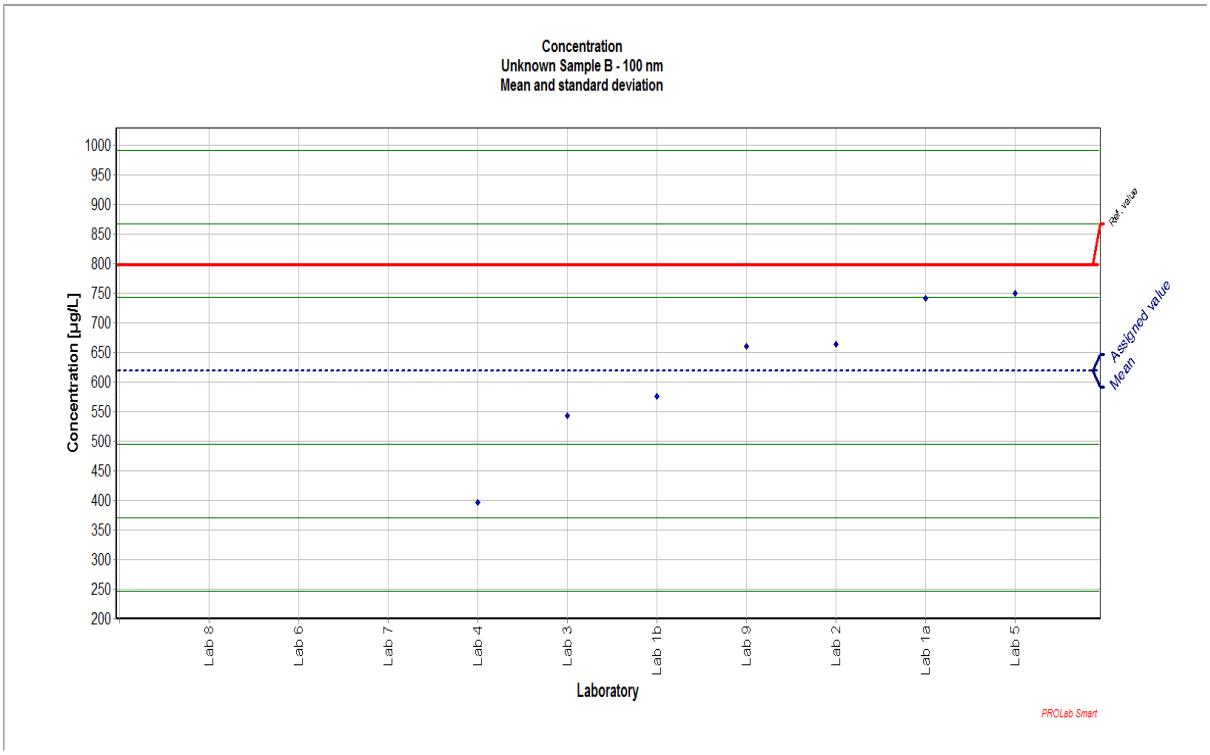

Figure 21 Unknown Sample B – AgNP(100nm)

8.2.5 Discussion of mean concentration values obtained

Table 8-3 shows the comparison between the mean concentration values obtained from the participating laboratories with the theoretical calculated value (Ref. Value, red line)

Table 8-3 Comparison of mean concentrations with reference values

	Mean Concentration [ng mL^{-1}]	Reference Concentration [ng mL^{-1}]	Difference
Unknown Sample A, 50nm	92.6	103	-10 %
Unknown Sample A, 100nm	554.9	798	-30 %
Unknown Sample B, 50nm	79.7	102	-21 %
Unknown Sample B, 100nm	618.9	795	-22 %

The following trends are observed:

- In the majority of cases, the concentrations determined within this exercise are lower compared to the reference values.

Based on indications given in the SOP provided, calibration/quantification was in almost all cases done post-channel against ionic silver. One laboratory however quantified both post-channel with ionic silver (Lab 1b) and pre-channel with particles (Lab 1a). Results (Table 8-4) show that pre-channel calibration lead to a much better apparent recovery rates indicating that some material loss occurs in the channel.

Table 8-4 Recovery rates in % comparing results calibrating pre-channel with particles and post-channel with ionic silver

	Post-channel calibration with ionic silver	Pre-channel calibration with particles
Unknown Sample A, 50nm	-26 %	+ 0.8 %
Unknown Sample A, 100nm	-21 %	+ 8 %
Unknown Sample B, 50nm	-43 %	- 29 %
Unknown Sample B, 100nm	-28 %	- 7 %

- The recovery for the 50 nm particles is better in “Unknown Sample A”

The reason for this might be attributed to the fact that “Unknown Sample A” has been dispatched as a 20 times more concentrated solution, which had to be diluted prior to analysis. Smaller particles in the diluted (ready-to-inject) “Unknown Sample B” may have undergone a partial dissolution. To minimise this effect in future exercises solutions should be dispatched as a concentrate which has to be diluted prior to analysis.

- The recovery for the 100 nm particles is slightly better in “Unknown Sample B”
- Recoveries for particles of 50 nm in size are better than for particles of 100 nm

This might be attributed to the peak shape of silver nanoparticles of 100 nm in size. The wide peak-broadening leads to less precise integration/quantification.

These four major trends are further supported statistically by calculating the z-scores.

8.2.6 Assessment of the performance of individual laboratories via z-scores

Z-Scores are based on the deviation of the laboratory mean value from an assigned value (in our case the absolute calculated concentration). The z-score values were calculated as follows-

Z scores = (Lab result – Theoretical Value) / s.d.

Z-scores calculated for this method validation exercise are shown in Figure 22.

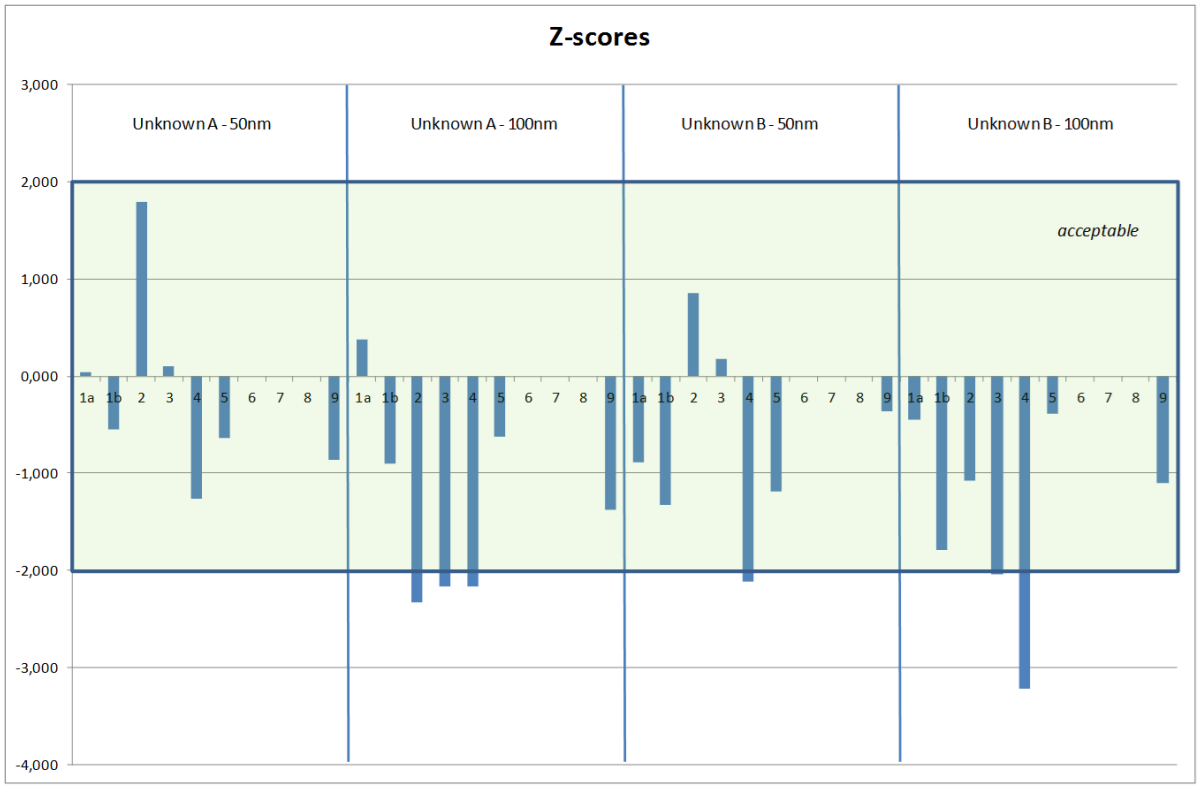

Figure 22 Z-scores for assessment of laboratories performance

8.2.7 Discussion of z-values

Values between -2 and +2 are considered “acceptable”. The lower the absolute z-score value, the closer the lab result is in relation to the theoretical value. Negative bars in Figure 22 indicate that the determined value is lower compared to the theoretical reference value. Positive bars indicate that

the determined value is higher compared to the theoretical reference value. The calculated z-scores confirm what has already been discussed previously.

8.3 Statistical evaluation of determined particle sizes

8.3.1 Reported values for size

Particle sizes determined by exercise-participants are shown in Table 8-5. The reference values for the two materials were the following: 52.4 nm and 99.4 nm

Table 8-5 Particle sizes determined for “Unknown Sample A” and “Unknown Sample B”

	Unknown Sample A (*)		Unknown Sample B (*)	
Laboratory Code	AgNP(50)	AgNP(100)	AgNP(50)	AgNP(100)
1	53	100	53	100
2	50.1	111.2	64.9	117.8
3	54	106	55	105
4	52	106	70	109
5	50	100	60	115
6	50	100	80	100
7	49.8	110.9	55	113.5
8	N/A	N/A	N/A	N/A
9	47	180	50	160

*Particle sizes are expressed in nm

8.3.2 Data distribution

Figure 21 to 24 show the distribution of results obtained for the determination of particles‘ size in “Unknown Sample A“ and “Unknown Sample B“. The dark blue lines represent the smoothed distribution of all test results while the light blue lines represent the cumulative distribution.

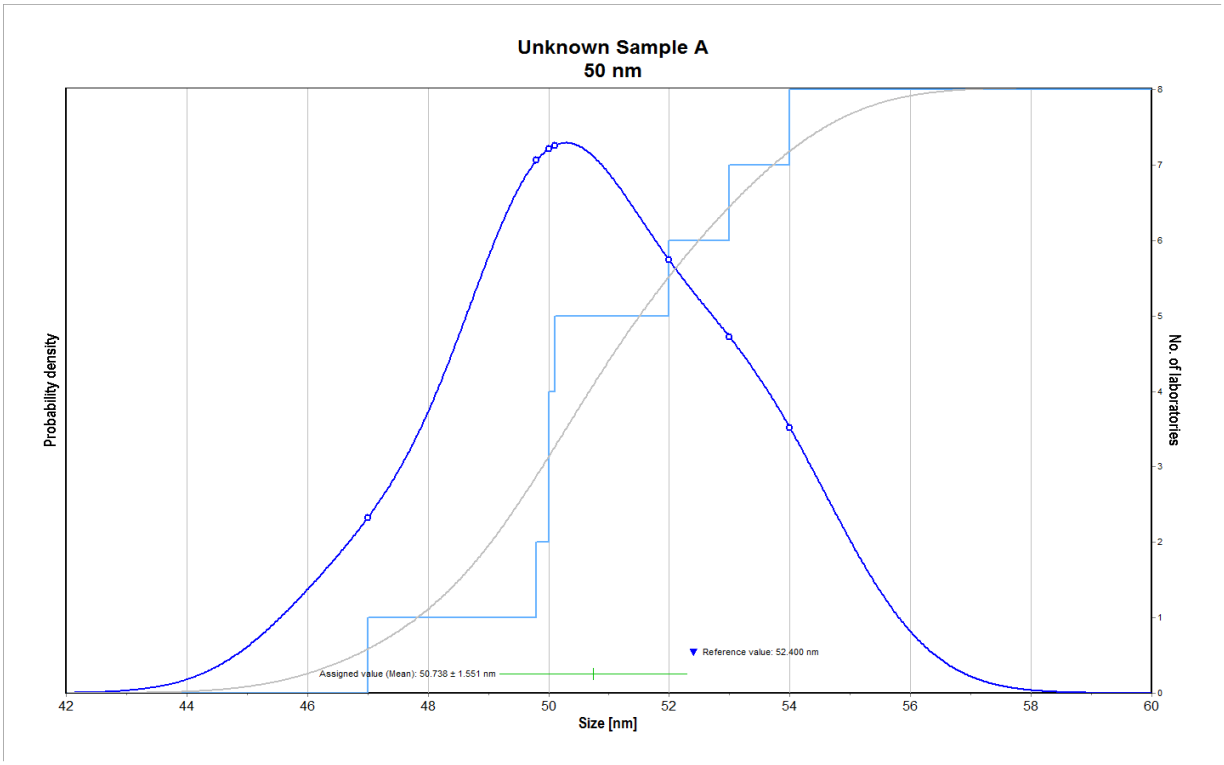

Figure 23 Data distribution for “Unknown Sample A”, 50nm

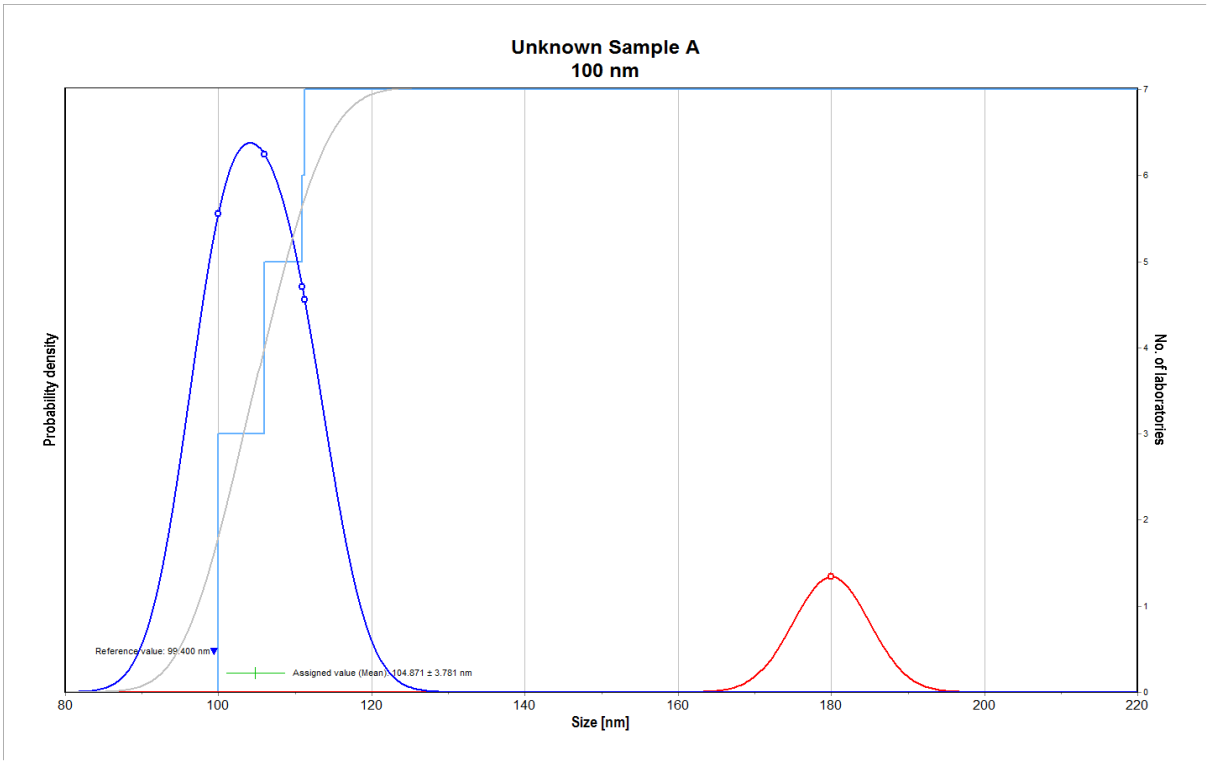

Figure 24 Data distribution for “Unknown Sample A”, 100nm

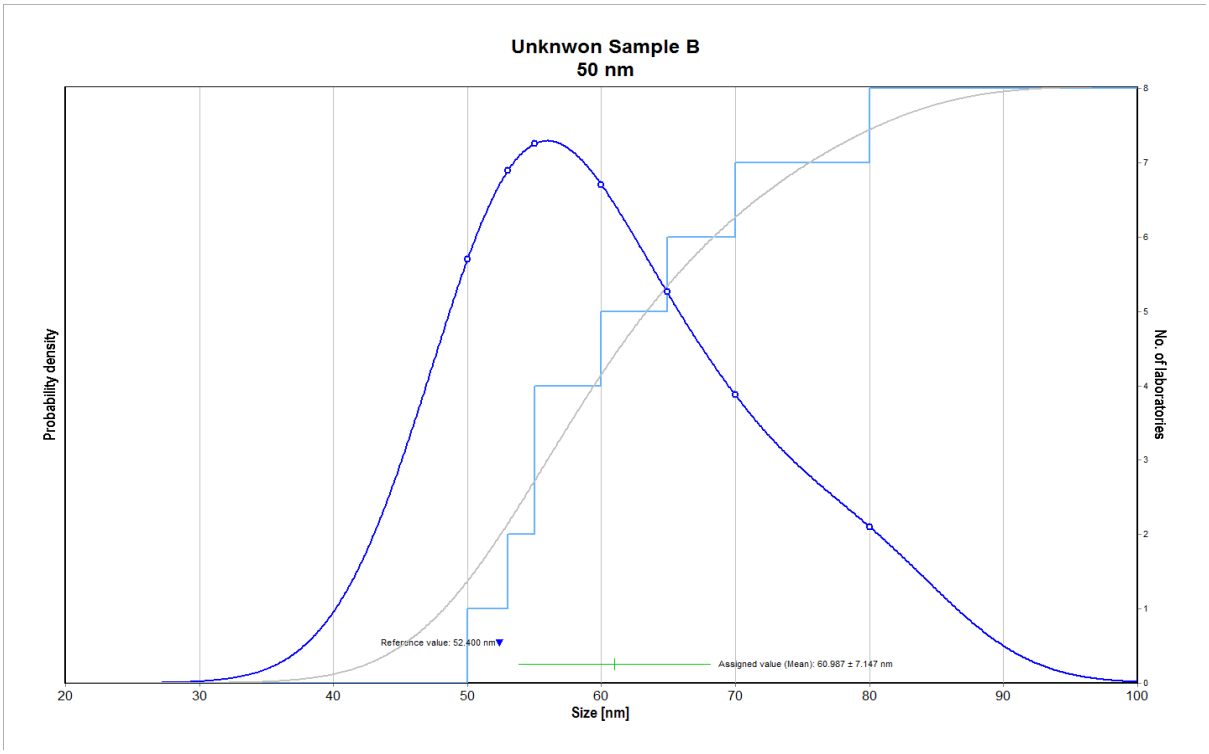

Figure 25 Data distribution for “Unknown Sample B”, 50nm

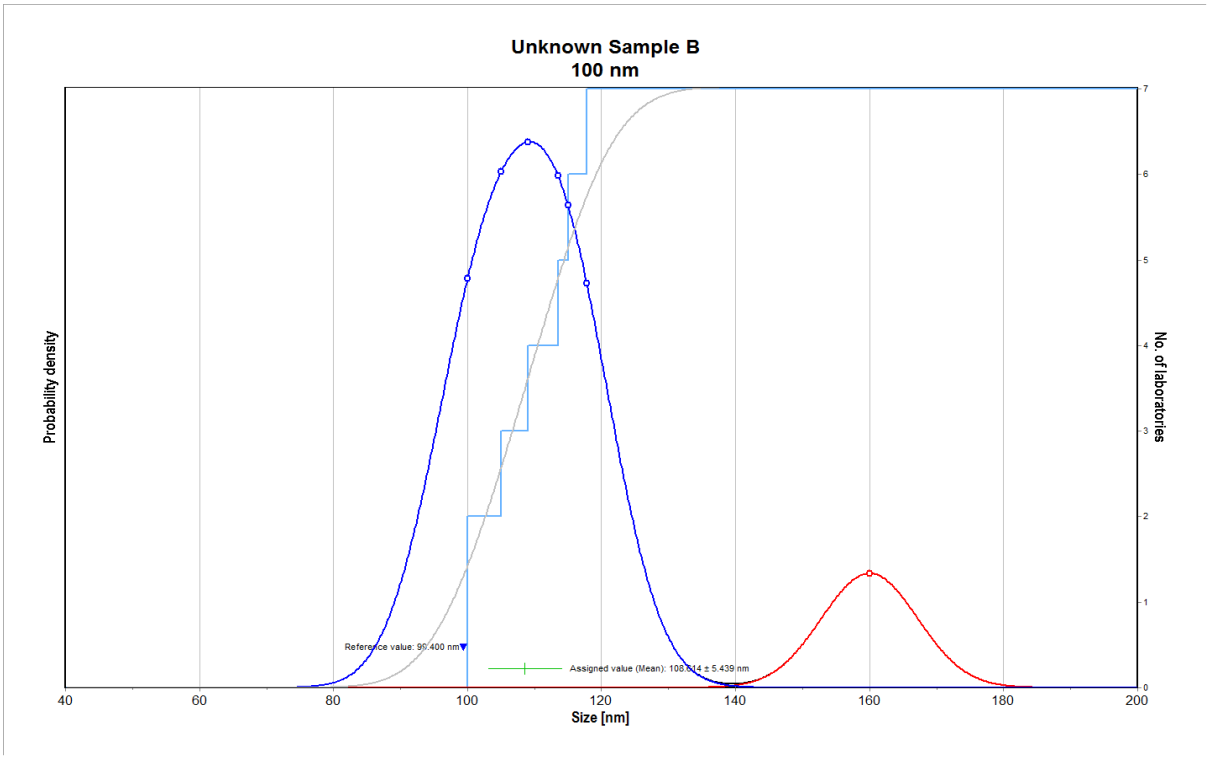

Figure 26 Data distribution for “Unknown Sample B”, 50nm

8.3.3 Outlier Tests

Data has been tested on outliers (Grubbs and Cochran). The following two values have been identified as outliers:

Lab 9, Unknown A, 100nm (data point = 180 nm)

Lab 9, Unknown B, 100nm (data point = 160 nm)

These two data-points have not been considered for the statistical evaluations.

8.3.4 Statistical Parameters according to ISO 5725-2

Results for some of the principal statistical parameters are shown in Table 8-6

Table 8-6 Results of selected statistical parameters

	Unknown Sample A		Unknown Sample B	
	50 nm	**100 nm**	**50 nm**	**100 nm**
Unit	nm	nm	nm	nm
No. of labs that submitted results	8	8	8	8
Mean	50.74	104.87	60.99	108.61
Reference Value	52.4	99.4	52.4	99.4
s.d.	2.194	5.002	10.108	7.195
Rel. s.d.	4.32	4.77	16.57	6.62
Limit of reproducibility, R(2.80 x sR)	6.144	14.006	28.303	20.147
Standard Error	0.776	1.891	3.574	2.720
Lower confidence limit of LPOD	49.186	101.09	53.84	103.175
Upper confidence limit of LPOD	52.289	108.653	68.135	114.054
Outliers	-	1 7 valid results	-	1 7 valid results

The following charts (Figure 25 to 28) represent the raw data for both samples/particle sizes, plotting laboratory-identifications on the x-axis and determined particle size on the y-axis. Values are plotted in ascending order. The blue dotted line represents the mean particle size, the green lines the standard deviations and the red line the theoretical reference value (the “real” value).

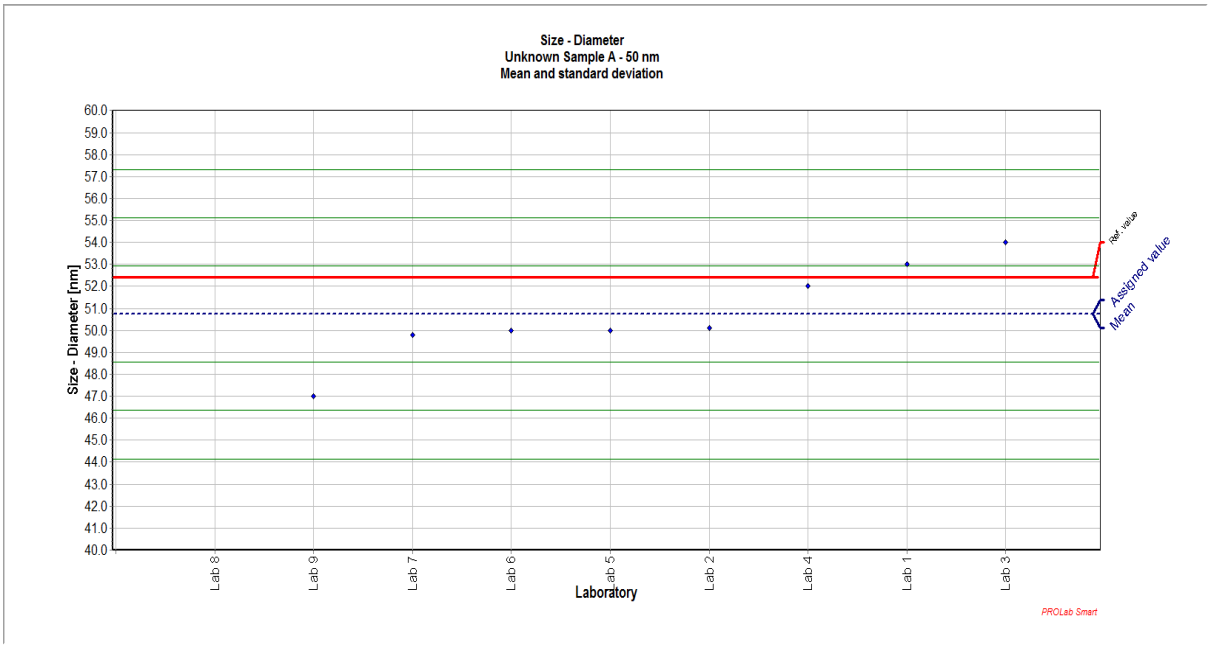

Figure 27 Unknown Sample A – 50nm

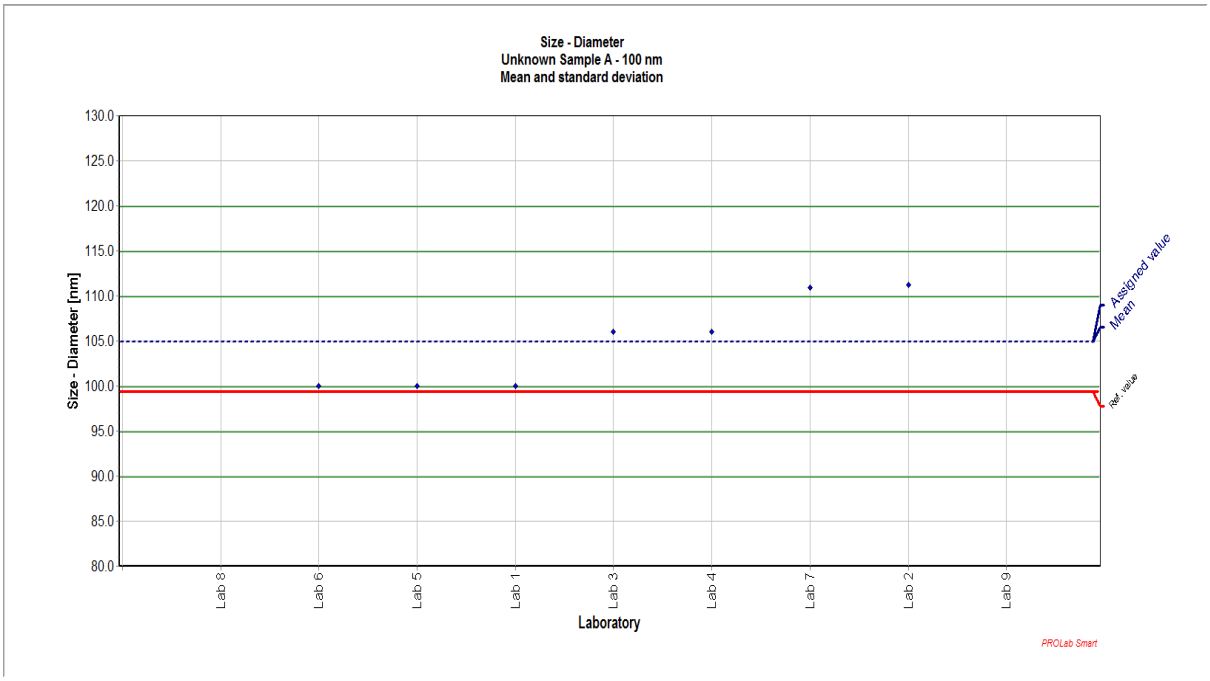

Figure 28 Unknown Sample A – 100nm

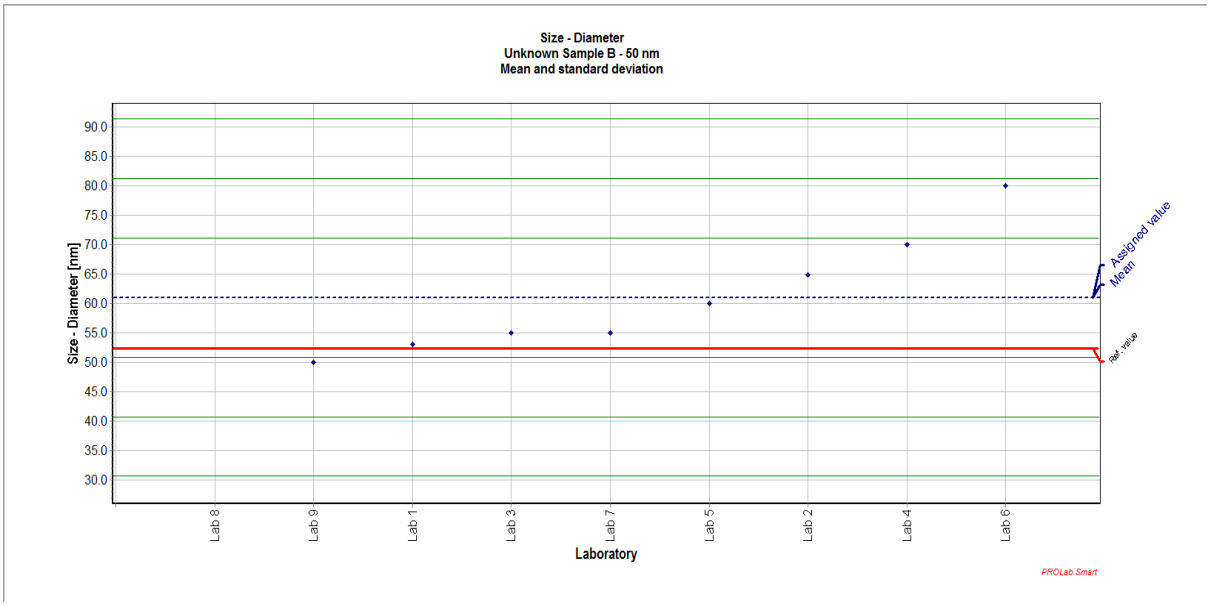

Figure 29 Unknown Sample B – 50nm

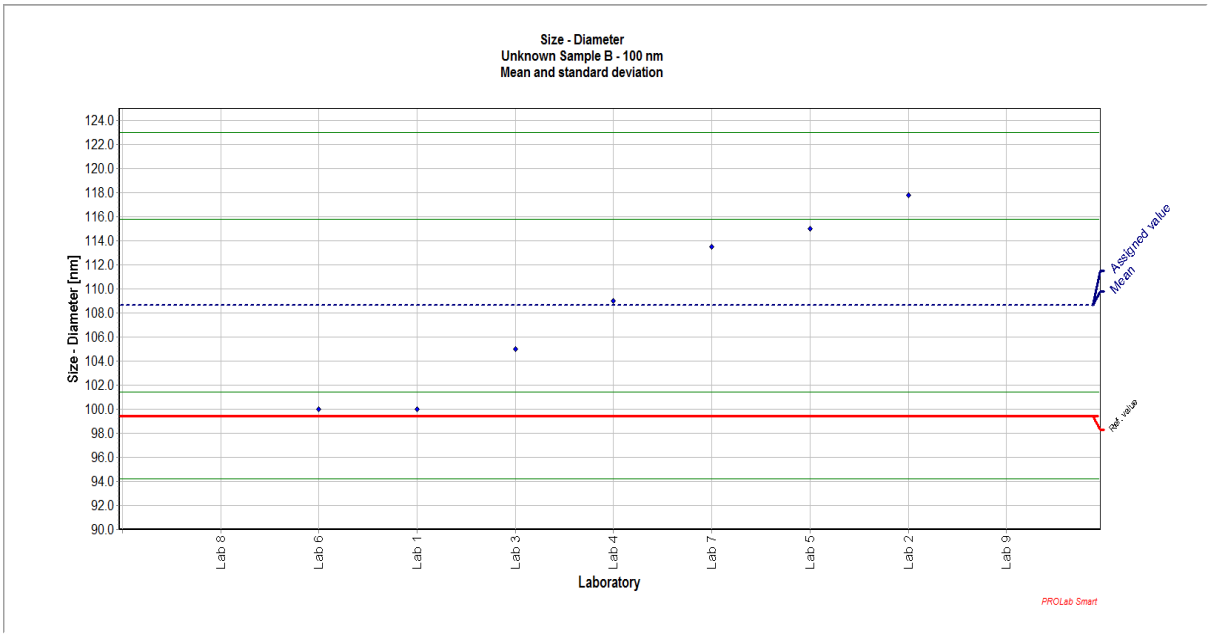

Figure 30 Unknown Sample B – 100nm

The comparison of the mean particle sizes of all results obtained by the participating laboratories with the theoretical value (Ref. Value, red line) is shown in Table 8-7.

Table 8-7 Comparison of mean particle sizes with reference values

	Mean Size [nm]	Reference Size [nm]	Difference
Unknown Sample A, 50nm	50.7	52.4	-3.2 %
Unknown Sample A, 100nm	104.9	99.4	+5.5 %
Unknown Sample B, 50nm	61.0	52.4	+16.4 %
Unknown Sample B, 100nm	108.6	99.4	+9.3 %

The following trends are observed:

- In 7 out of 8 cases, the size determined for “Unknown Sample B, 50nm” is higher compared to the same particle type in “Unknown Sample A”
- The size determined in “Unknown Sample B, 50nm” is around 10nm too high.
- The size determined in “Unknown Sample A, 50nm” is very accurate
- The size determined in “Unknown Sample A, 100nm” is sufficiently accurate
- The size determined in “Unknown Sample B, 100nm” is less accurate compared to “Unknown Sample A” of the same size

In general, the results indicate that particles in the ready-to-inject sample (Unknown Sample B) have undergone an increase in diameter. The reason might be attributed to a less stable colloidal suspension which has undergone some degree of aggregation. In any future exercises the samples should be dispatched as a concentrate which needs to be diluted prior to analysis.

The five major trends listed above are further supported statistically by calculating the z-scores.

8.3.5 Assessment of the performance of individual laboratories via z-scores

Z-Scores are based on the deviation of the laboratory mean value from an assigned value (in our case the absolute declared particle size).

Calculation z-scores:

Z scores =	(Lab result – Theoretical Value) / s.d.

Z-scores calculated for this method validation exercise are depicted in Figure 31

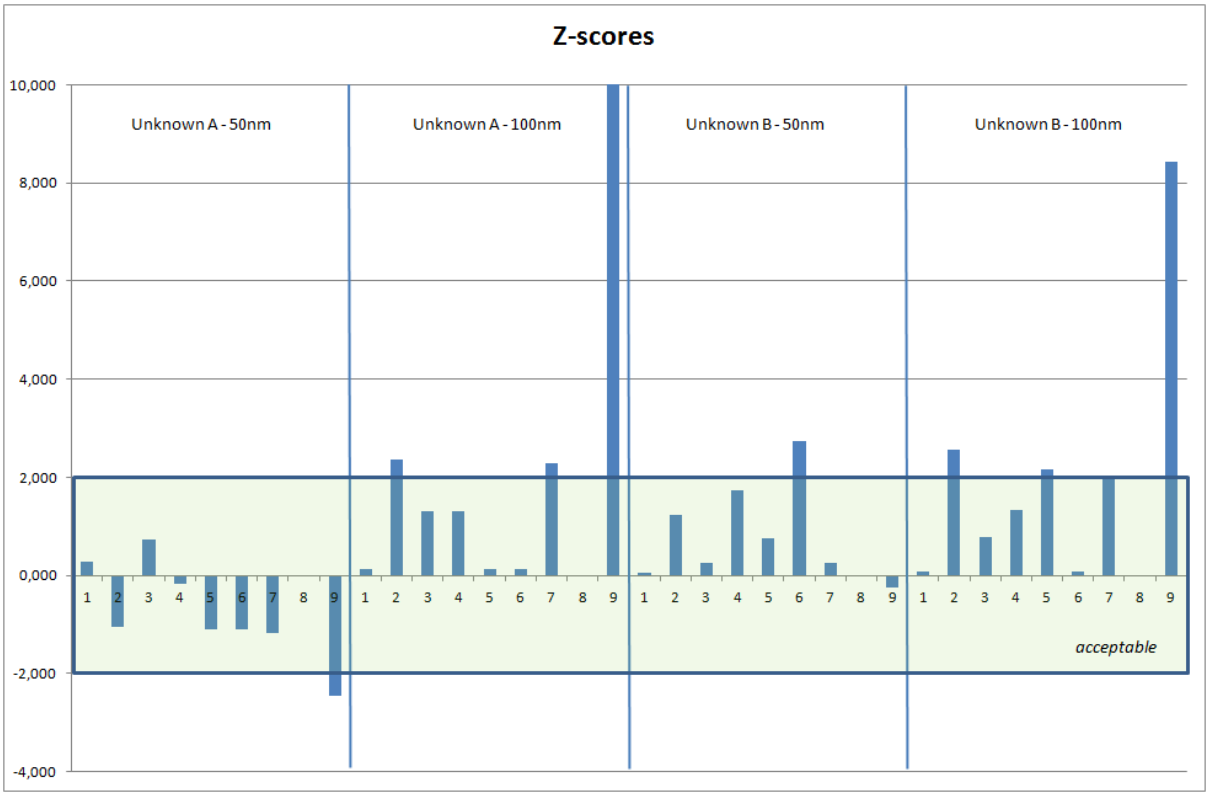

Figure 31 Z-scores for assessment of laboratories performance

8.3.6 Discussion of z-scores

Values between -2 and +2 are considered “acceptable”. The lower the absolute z-score value, the closer the lab result is in relation to the theoretical value. Negative bars in figure 18 indicate that the determined value is lower compared to the theoretical reference value. Positive bars indicate that the determined value is higher compared to the theoretical reference value.

The calculated z-scores confirm what has already been mentioned in the previous sections

Size determination for the 100 nm particles done by laboratory 9 in both “Unknown Samples A & B” have by far exceeded the reference values. The reason should be further investigated.

8.4 Concluding Remarks

The proposed methodology was assessed in an unbiased manner by asking participants to analyse two “unknown” samples each. One sample had to be diluted before analysis and the other was ready to inject. The diluted and the ready-to-inject sample were composed of the same particles and had the almost the same final concentrations of silver. Concentrations of the two particle sizes were adjusted to represent a “borderline” case in regard to the definition of nanomaterials (50% of the number of particles < 100 nm and 50% above 100 nm).

Results obtained in this restricted method performance exercise have provided the data necessary to make a preliminary assessment of the precision achievable with the technique. Table 8 lists the theoretical values along with the measured mean value and their respective standard deviations for “Unknown Sample A” which yielded better results.

Table 8-8 Theoretical values compared to measured mean values and their respective standard deviations obtained in this exercise (Only “Unknown Sample A”).

	Concentration [ng mL^{-1}]			Particle Size [nm]		
	Theoretical Value	Mean Value	S.D.	Theoretical Value	Mean Value	S.D.
“Unknown Sample A” 50nm	103	92.7	± 46.8	52.4	50.9	2.2
“Unknown Sample A” 100nm	798	554.9	± 182.6	99.4	104.3	4.9

Trueness (accuracy) and especially precision of the quantification does not allow a clear identification of whether a particle mixture is or is not a nanomaterial under borderline conditions according to the definition. The accuracy and precision of particle size-determination however are satisfactory.

Table 8-9 shows how accuracy and precision of concentrations determined in this exercise influence the identification/non-identification of a nanomaterial by converting mass concentrations into the number of particles.

Table 8-9 Conversion of measured mass concentrations into corresponding number of particles

Case	cAgNP50 [ppb]	cAgNP100 [ppb]	Ratio #(AgNP50)/ #(AgNP100)	#(AgNP)<50nm [%]	Nanomaterial according to definition?
Theoretical Concentrations	103	798	1.03	51	Yes
Mean Concentration of current method performance exercise	92.7	554.9	1.34	57	Yes
1st extreme AgNP50nm Mean Conc. +SD AgNP100nm Mean Conc. -SD	139.5	372.3	3.00	75	Yes
2nd extreme AgNP50nm Mean Conc. -SD AgNP100nm Mean Conc. +SD	45.9	737.5	0.50	33	No

How could the methodology be improved?

- The use of pre-channel calibration with silver nanoparticles has shown to be able to significantly improve precision and trueness. The use of silver particles for this type of calibration is however limited by the lack of availability of such particles across the necessary size range and the absence of certified materials (certified for size and concentration).
- In the current exercise not all participating laboratories used exactly the same instrumental setup. Most used an online combination of AF4-ICP-MS. Some however did use other combinations. The method's performance is likely to increase if all laboratories use exactly the same instrumental setup.

9 Future work

The study which has been detailed in this report was undertaken as a first stage evaluation of AF4-ICPMS methodologies for measuring nanoparticle mass and number size distributions. The results have shown the basic separation methodology to be promising but the mass quantification by ICP-MS has shown less than ideal behaviour. From this study it has been possible to identify number of key areas for improvements as detailed below. These improvements cover not only the basic practice of the separation process but also the stability of the particles under test and the membrane materials used in the separation.

9.1 Dynamic range of AF4 separations

The study has shown that the separation methodology is relatively effective and reproducible over the range of particle sizes considered in this study i.e. 10nm to 100nm. For the particular application considered in this work, Ag nanoparticles, this range would normally cover a large portion of the size range relevant to silver based nanomaterials but for strict application to the definition this range is not sufficiently large.

a) Lower limit: The method has been developed to ensure that there is void peak separation of particles to below 10nm but as the definition requires information on size distributions to 1nm. It is likely that this issue could be resolved by increasing the analysis cross-flow but in order to reach 1nm the high cross-flow required is likely to result in loss of larger particle by absorption onto the accumulation wall

b) Upper limit: The current verified upper limit of around 110-120nm could be extended by decreasing the cross-flow but this would likely lead to the loss of the smaller particles into the void peak.

The most likely way of resolving these two contradictory problems would be to analyse each sample using 2 or 3 different elution profiles each of which has been specifically optimised to operate efficiently in different specific narrower but overlapping size ranges. Eg. (1nm-60nm) and (30nm to 300nm) and (50nm to 500nm).

9.2 Quantification ICP-MS

In the study is apparent that the quantification of the silver mass by ICP-MS did not give adequately accurate or reproducible results. The origin of these results is likely due to a combination of several issues including the lack of any single standardised calibration method, variability in column recovery and also the possible degradation of the samples particularly when the participating laboratories had not been able to respect the time scale requested.

Approaches to improving these problems could the following

a) Specify a more detailed methodology for the calibration of the ICP-MS system with respect to the AF4 column. One method could be the use of pre-column injection of nanoparticle standards which has been found by JRC to give the most accurate and reproducible method. This method, though technically promising, has the disadvantage that it is bound by the availability of accurately characterised standards of the same/similar materials to the samples being analysed. These requirements may be difficult to satisfy in the real-world situation particularly when having to analyse other samples from families of materials where not even pseudo-standards can be sourced.

b) In the case where pre-column injection of nanoparticles is not used calibration/quantification using isotope dilution method would be a powerful method to resolve some of the difficulties with silver nanoparticle analysis by ICP-MS. This method could be implemented for silver but for other types of nanomaterials its application would depend on the availability of suitable, stable isotope materials.

c) The use of more chemically stable silver nanoparticles (eg. stabilised by PVP or PEG) would permit further evaluation studies to be conducted with less influence of materials degradation. This would be relevant only to ring-trial studies and would not be a solution to the analysis of unknown silver samples.

9.3 Applicability of the method to alternative types of silver particles

The ILC has concentrated very specifically on one common type on silver nanoparticle, citrate stabilised, but this is only one of several types likely to be found in commercial products. Under ideal condition the use of AF4 requires that there be no significant electrostatic or chemical interaction between the particle and the accumulation wall. Unfortunately this is difficult to ensure in practice as it would be undertake a detailed optimisation process for each new type of particle. This problem is one of the most difficult which must be tackled

a) A partial solution to the problem would be to verify the correct functioning of the existing method for other common types of particle stabilisation such as PEG, PVP, tannic acid and other neutral/negatively charged stabilizers.

b) In the case that the existing method proves to be insufficiently generic it would be necessary of search for alternate conditions of membrane/eluent/pH/surfactant which can more widely applied.

9.4 Application of DLS for on-line nano-particle sizing

A major issue in developing the methods for simultaneously quantifying materials mass and size is that particle size determination depends on having suitable standards and the assumption that any calibrations standards will behave like the actual analyte particles. In this study this was the case but in real-world testing this is more difficult to guarantee. The ideal solution to this would be to

eliminate the need for particle size standards and obtain information on particle sizes by direct measurement online by DLS. This solution, although elegant does have the problem of the sensitivity of DLS when analysing very small or poorly scattering particles types. The implementation of this solution would require careful examination of the materials concentrations required to obtain acceptable DLS performance in the particle size range of 1-20nm.

9.5 Mass-number distribution conversion: reliability of methodology

The ultimate goal of this activity is to arrive at a number size distribution by mathematical conversion of data in the form of a mass-size distribution. This is a process which may introduce large errors as particle size approaches the lower limit of the definition and it will be very important to quantify the level of errors in respect of the requirement of the definition.

9.6 Control of eluent pH

The eluent which was recommended in this work was high purity water modified only with a small amount of NaOH to adjust the pH to 9.4 with additional stabilising buffer being added. One participating laboratory did note that during a day of measurement the pH of the eluent solution decreases excessively (by absorption of atmospheric CO_2) and that this could modify the retention times. Although the variation of retention time with pH has been noted during method development, in practice, the typical change the pH found (<0.2 pH units in 2 litres of fresh eluent) in the JRC laboratories was insufficient to create any problem and so was not taken into consideration in the SOP. In future work it may be advisable to avoid the risk of this problem by considering the addition of a small quantity of a buffer agent to counteract the effect of CO2 absorption. A suitable reagent for this purpose would be a small quantity (0.1-0.5mM) of $(NH_4)_2CO_3$ as discussed in the work by Loeschner et al [7].

9.7 Quality control of membranes

One final issue which has been noted in this study is that membrane quality can have a strong influence on the effectiveness of the separations achievable. This is a problem which can only be resolved by the materials suppliers but for the purposes of future study this problem should be avoided by supplying participants with materials from a single batch of membranes.

10 Overall summary and conclusions

The Nanobiosciences and Chemical and Testing Units of the Joint Research Centre's Institute for Health and Consumer Protection have been collaborating on the development of methods for measuring nanoparticle number size distributions in support of the implementation of the European commission recommended definition of a nanomaterial.

As part of this activity a method which combines a particle size separation (Asymmetric Flow Field Flow Fractionation (A4) step and a particle detection/quantification step (Induction Coupled Plasma-Mass Spectrometry (ICP-MS)) has been examined and optimised experimentally for use in the analysis of aqueous dispersed silver nanoparticles. Following an internal validation, the method has been documented in the form of a Standard Operating Procedure (SOP) designed to provide all the necessary information to allow the method to be applied by suitably equipped external laboratories. To verify the transferability of the method an international ring-trial was organized by JRC in which 8 independent laboratories were provided with detailed documentation and suitable test materials to allow them to test the transferability of the SOP.

The results obtained have led to the following main conclusions

1) The separation methodology, when applied with the reference standards was found to be transferable to different laboratories with all laboratories being able to separate the recommended mixtures of mono-dispersed materials. A possible improvement to the separation methodology which may be considered would be the addition of a low concentration of a pH buffer to the eluent.

2) In the course of the study it was found that two laboratories reported problems of separation resulting from the quality of the separation membranes. Substitution of membrane with that from alternate batches or alternate manufacturers was found to resolve the problem. It should be noted that during method development this problem has also been observed by the organizing laboratory (JRC) although during the laboratory trial the problem was not observed.

3) The separation methodology, when applied with the "unknown samples A and B" and using UV absorption detection showed five of nine laboratories as being able to clearly separate the bimodal mixture. Three of the nine laboratories were able to obtain elution curves which showed evidence of the two peaks but with poor signal/noise ratio. Only one laboratory was not able to show evidence of the bimodal mix.

4) A statistical analyses of the result obtained from the unknown samples showed that acceptable accuracy and reproducibility was obtained for the measurement of nanoparticle size but that the quantification of the amount of silver using the ICP-MS was not sufficiently accurate or reproducible.

5) Sample stability: It is known that silver nanoparticles often exhibit problems of stability and may suffer from aggregation or dissolution during long term storage. A number of the participants were not able to complete their analyses in the recommended time period and it is likely that this may have influenced negatively on the quality of results obtained.

It should be noted that prior to initiating the inter-laboratory trial a series of studies were conducted by JRC to evaluate the useful shelf life of the nanoparticle mixtures. The results showed that dilute mixtures similar to the unknown samples A and B when correctly stored do not change more than 10% over 4 weeks. Since the test recommended that measurement be conducted within 4 weeks of receiving the material no major deterioration in the samples was expected. To further verify this a series of additional samples prepared together with those used in the ring trial were stored at JRC and analysed periodically during the 4 week time period specified for the study. The results obtained confirmed that no serious deterioration of the pseudo-standards or the unknown samples A/B samples had occurred over the period of the trial. It should be noted that this evaluation does not take into account the possible deterioration during transport or as result of non-ideal storage and handling.

11 References

1. European Parliament. Strasbourg 2009. European Parliament resolution of 24 April 2009 on regulatory aspects of nanomaterials (2008/2208(INI)). P6_TA(2009)0328 Nanomaterials. Available at http://www.europarl.europa.eu/sides/getDoc.do?type=TA&reference=P6-TA-2009-0328&format=XML&language=EN.
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6. Karl-Gustav Wahlund, Flow field-flow fractionation: Critical overview. Journal of Chromatography A, 1287 (2013) 97– 112.
7. Optimization and evaluation of asymmetric flow field-flow fractionation of silver nanoparticles Journal of Chromatography A, Volume 1272, 11 January 2013, Pages 116-125 Katrin Loeschner, Jana Navratilova, Samuel Legros, Stephan Wagner, Ringo Grombe, James Snell, Frank von der Kammer, Erik H. Larsen

Annex 1: Elution curves of 40nm:60nm mix

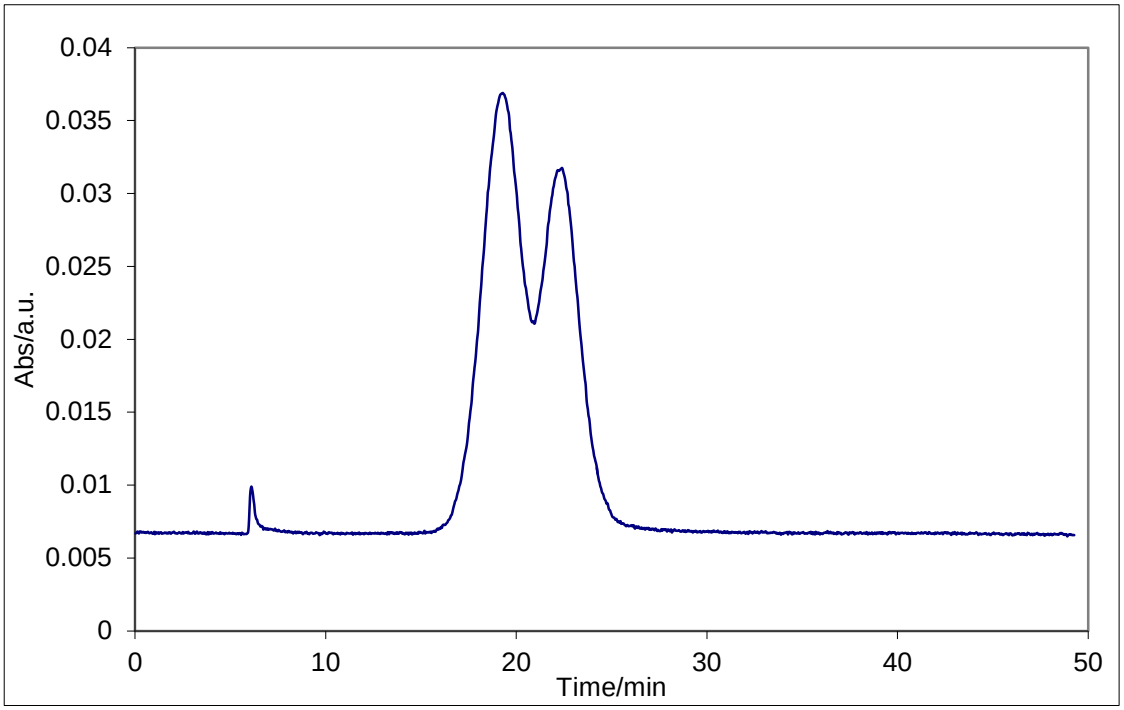


Figure 32 Laboratory 1 Elution curves of 40nm:60nm mix

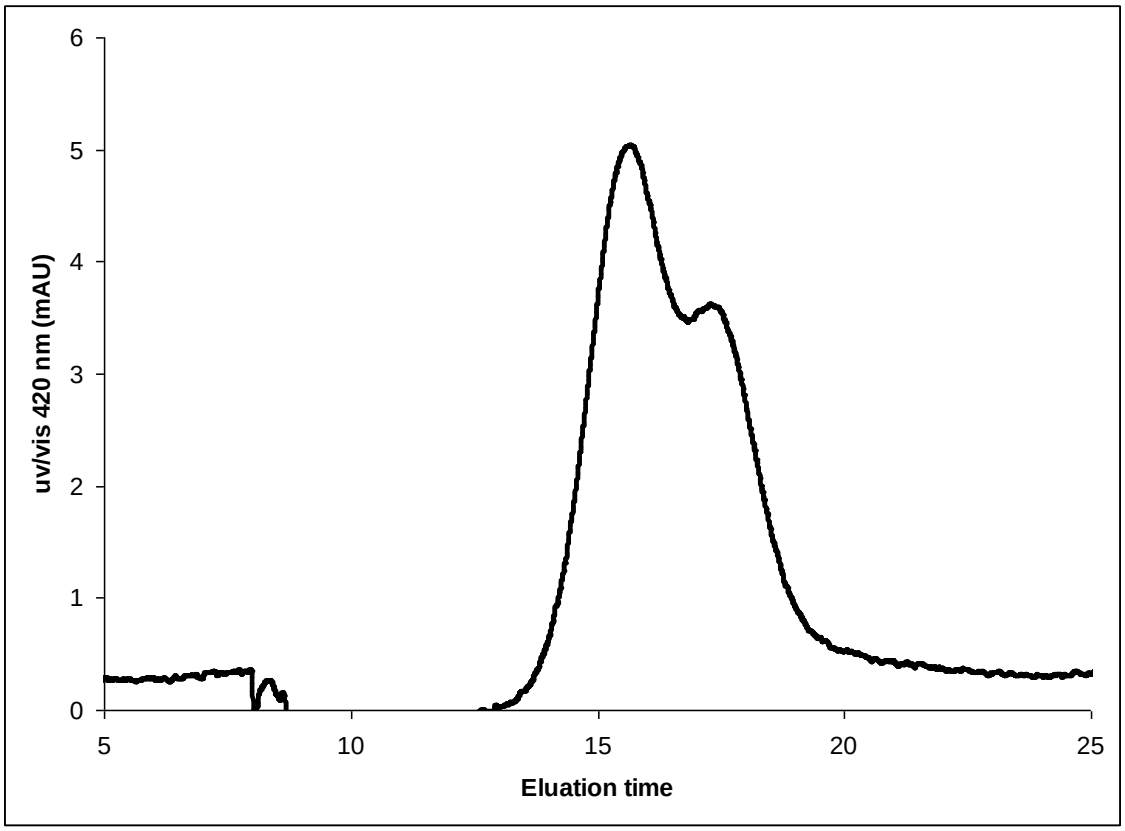


Figure 33 Laboratory 2 Elution curves of 40nm:60nm mix

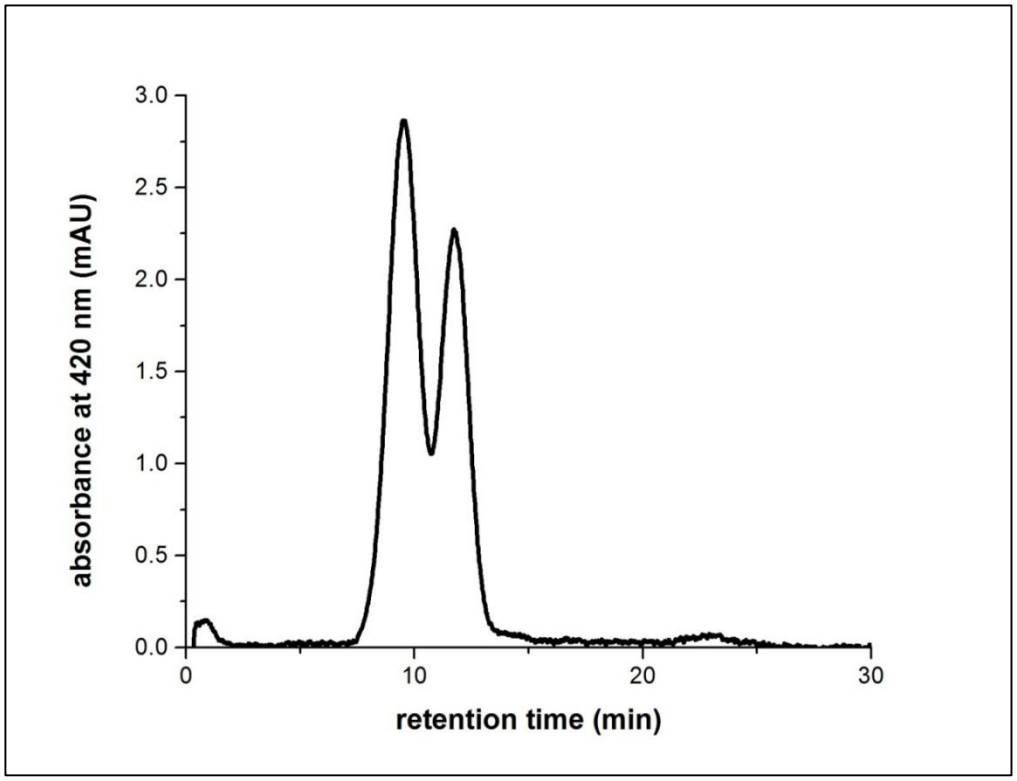


Figure 34 Laboratory 3 Elution curves of 40nm:60nm mix

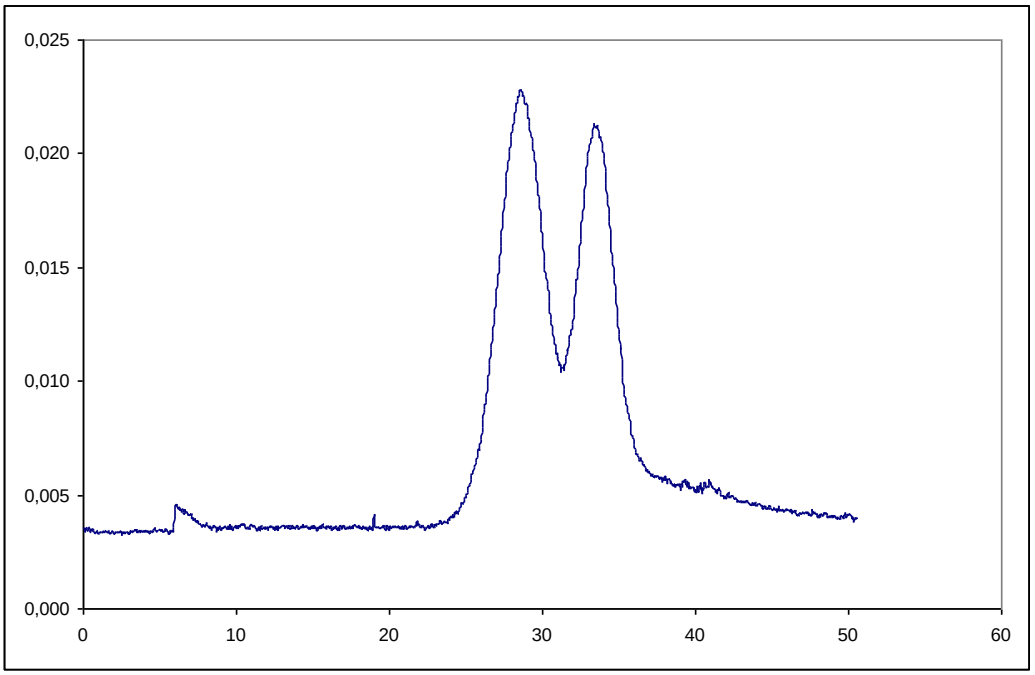


Figure 35 Laboratory 4 Elution curves of 40nm:60nm mix

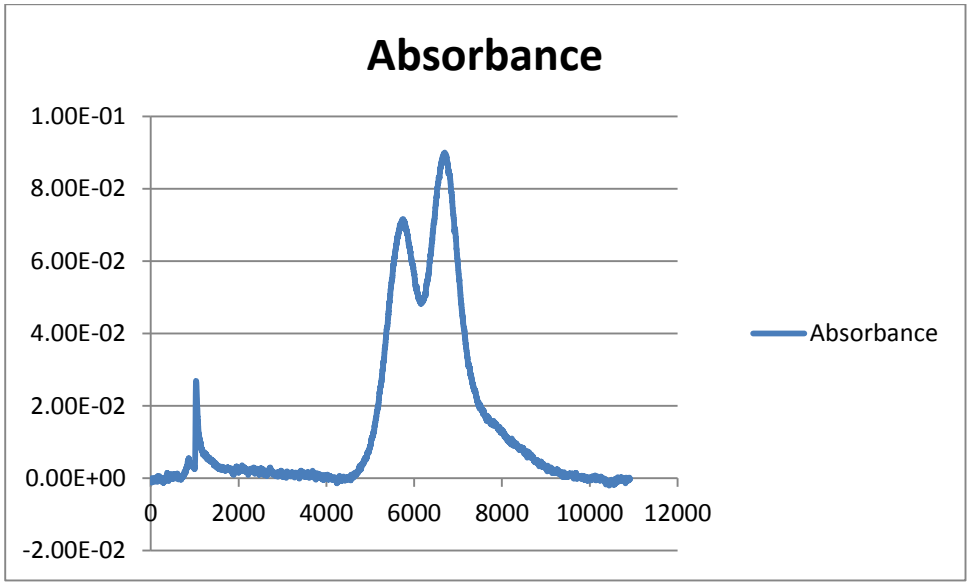


Figure 36 Laboratory 5 Elution curves of 40nm:60nm mix

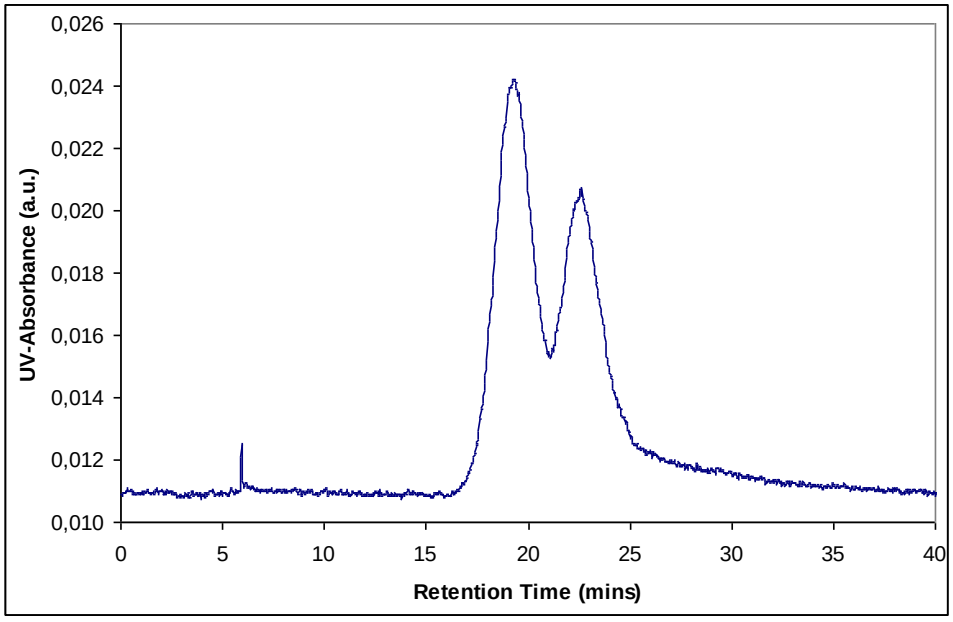


Figure 37 Laboratory 6 Elution curves of 40nm:60nm mix

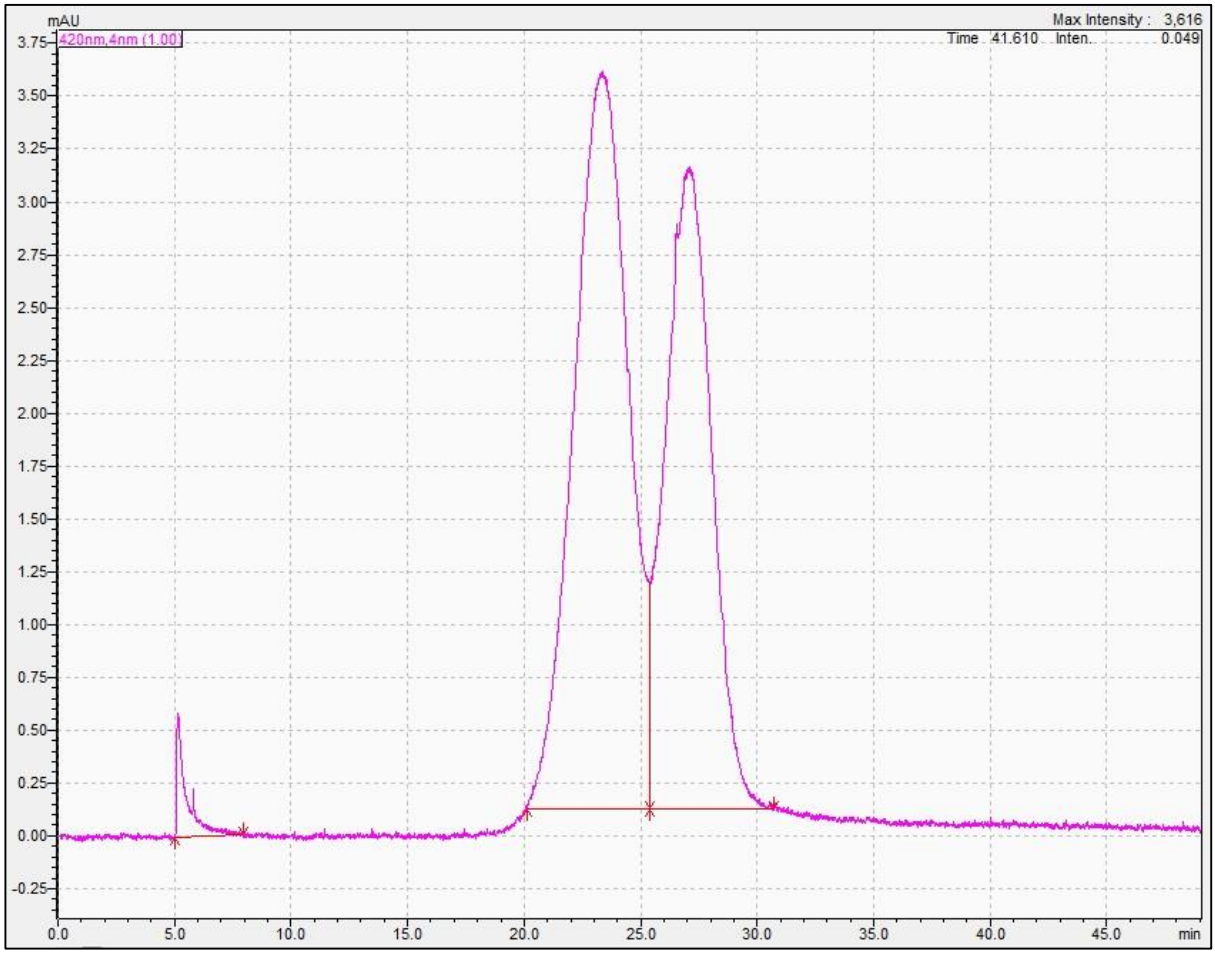


Figure 38 Laboratory 7 Elution curves of 40nm:60nm mix

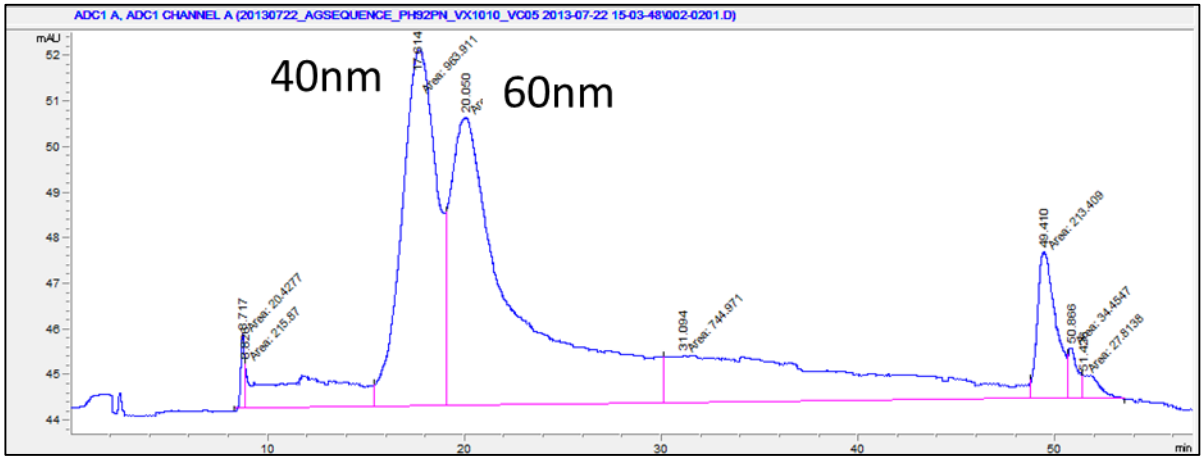


Figure 39 Laboratory 8 Elution curves of 40nm:60nm mix

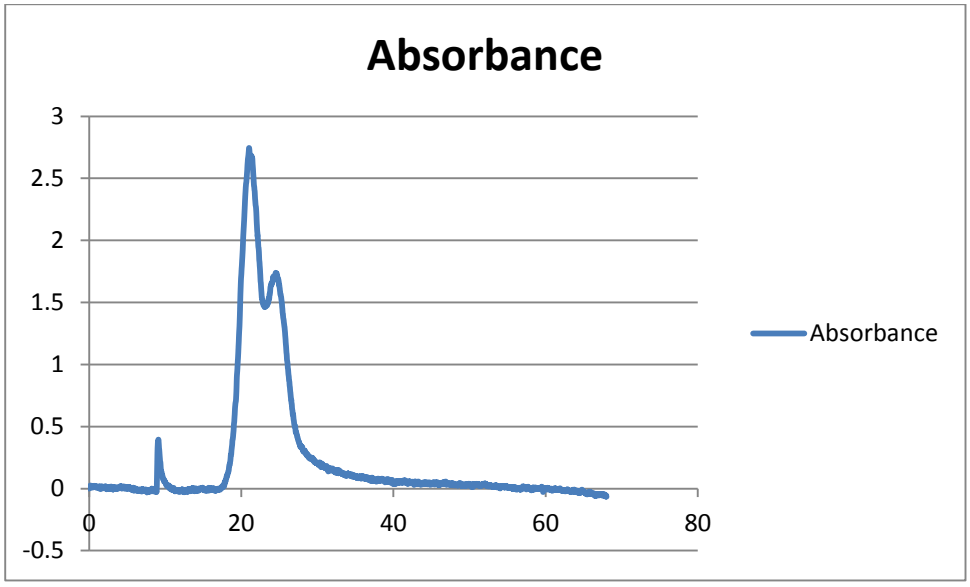


Figure 40 Laboratory 9 Elution curves of 40nm:60nm mix

Annex 2: Elution curves from UV and ICPMS of unknown samples A and B

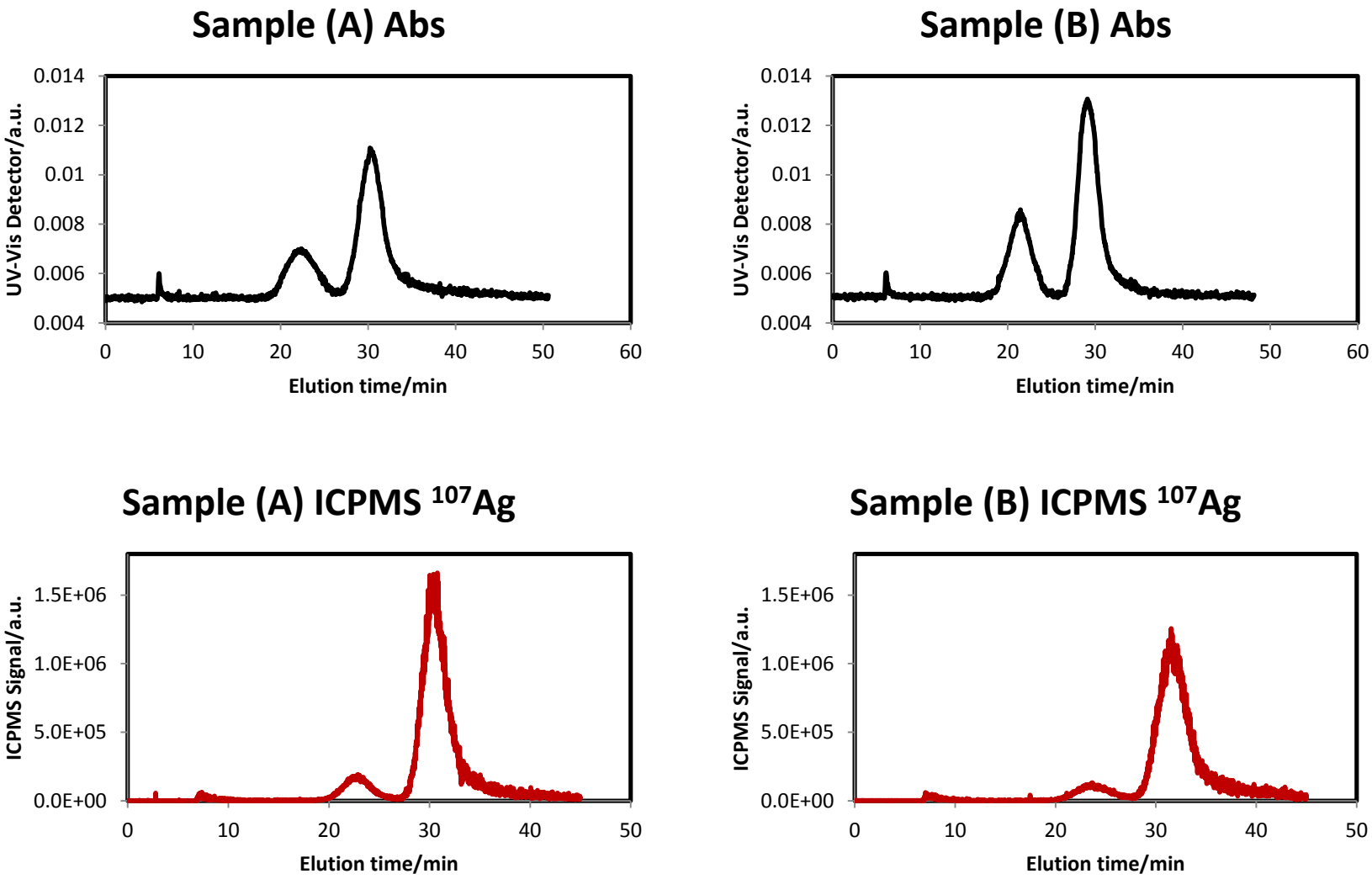


Figure 41 ICP-MS and UV Results from Lab 1

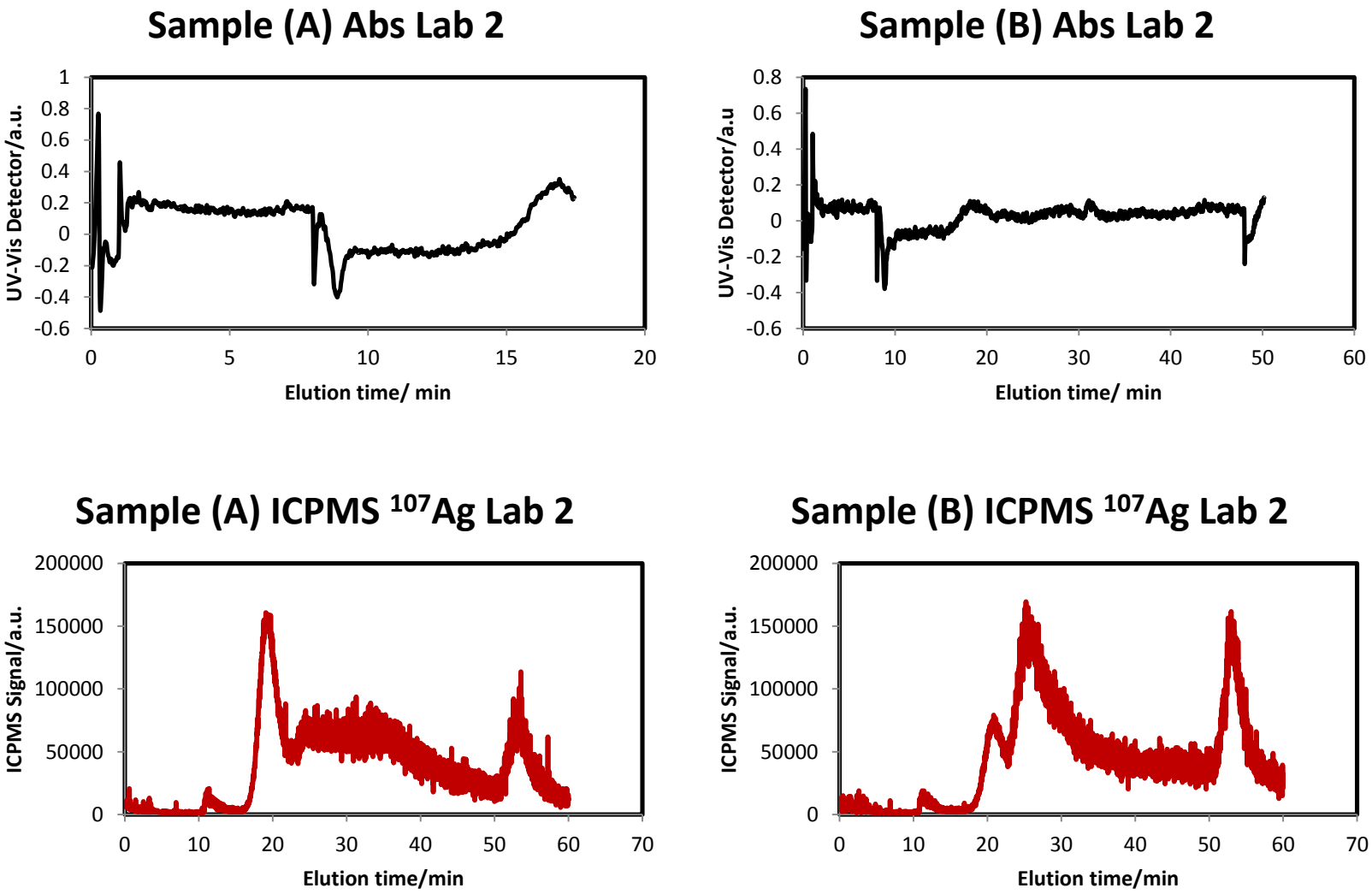


Figure 42 ICP-MS and UV Results from Lab 2

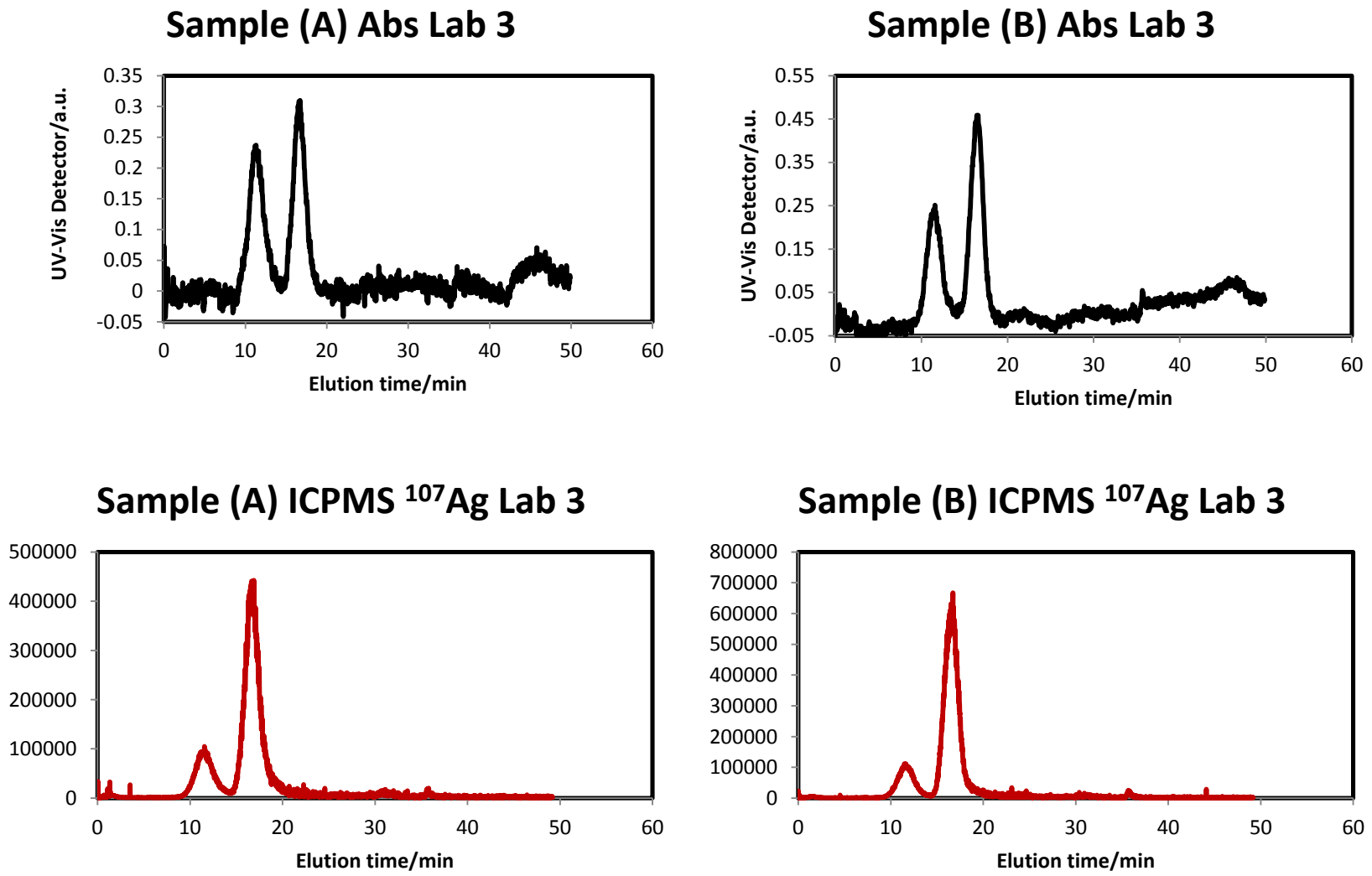
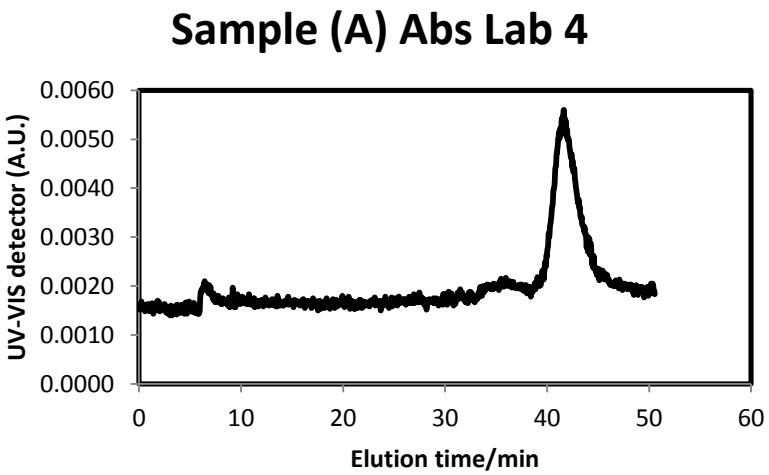
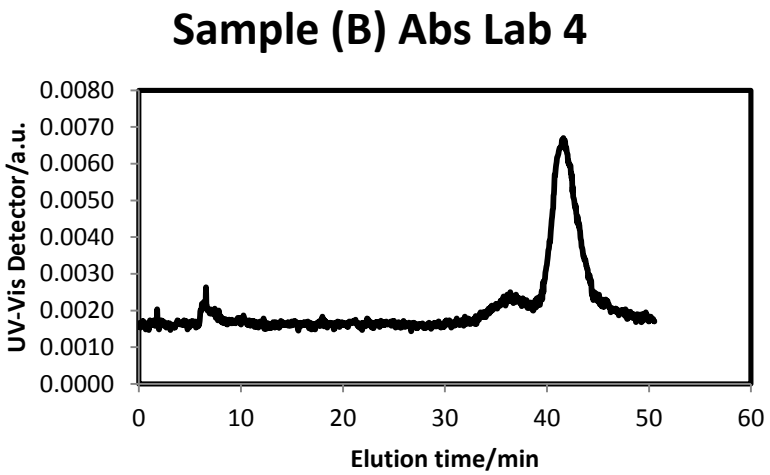
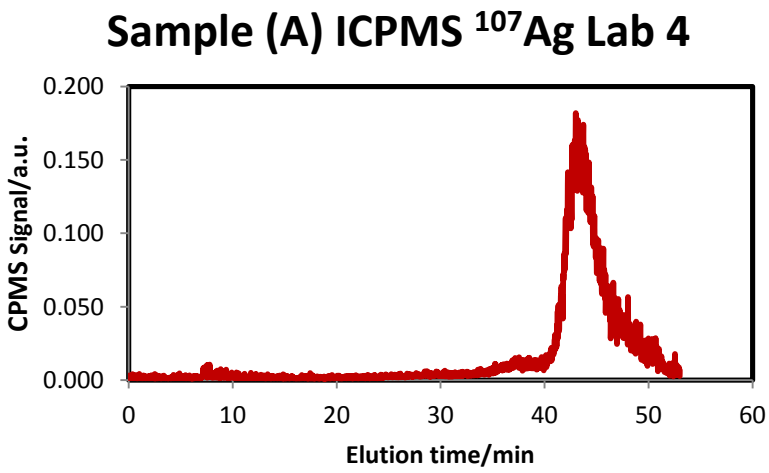


Figure 43 ICP-MS and UV Results from Lab 3







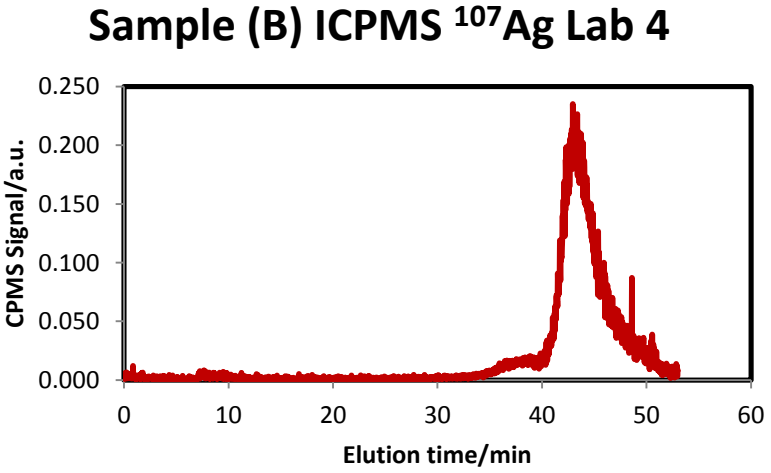
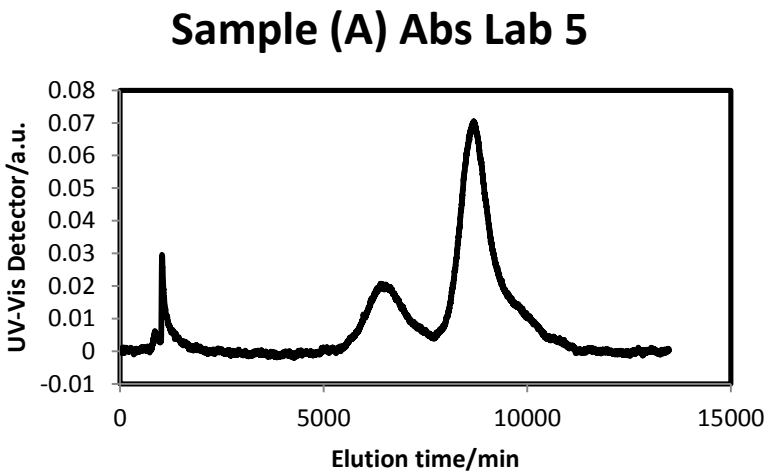


Figure 44 ICP-MS and UV Results from Lab 4



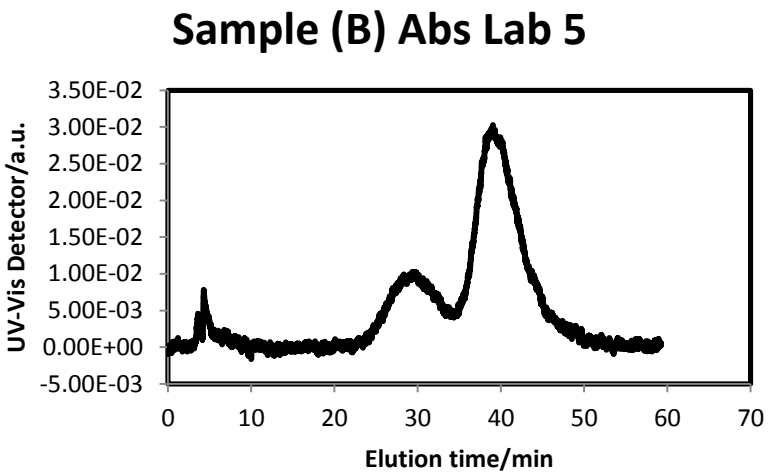
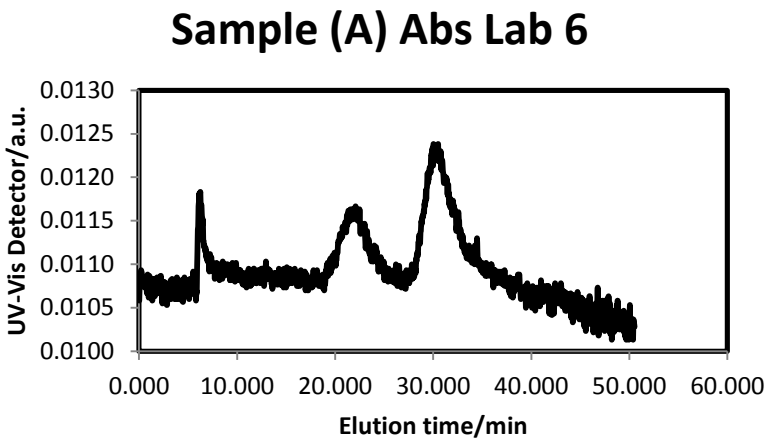
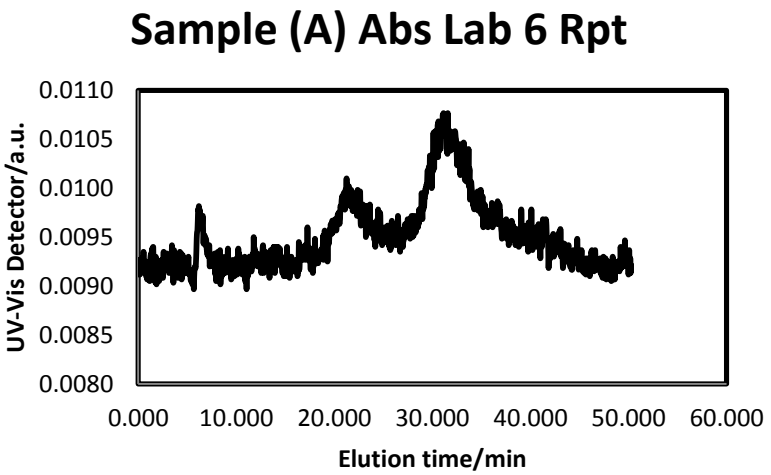
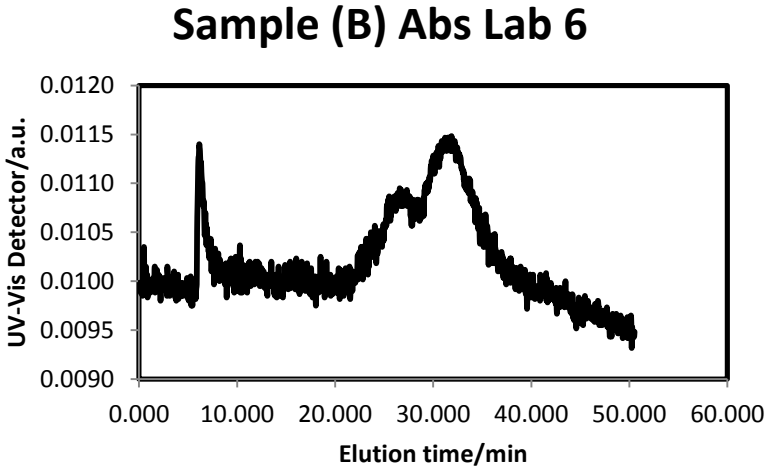


Figure 45 UV Results from Lab 5 ICP-MS DATA not available







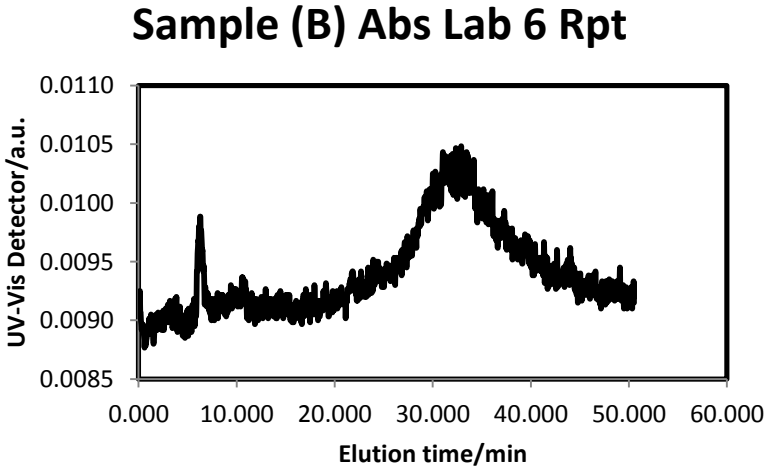


Figure 46 UV Results from Lab 6 ICP-MS DATA not available

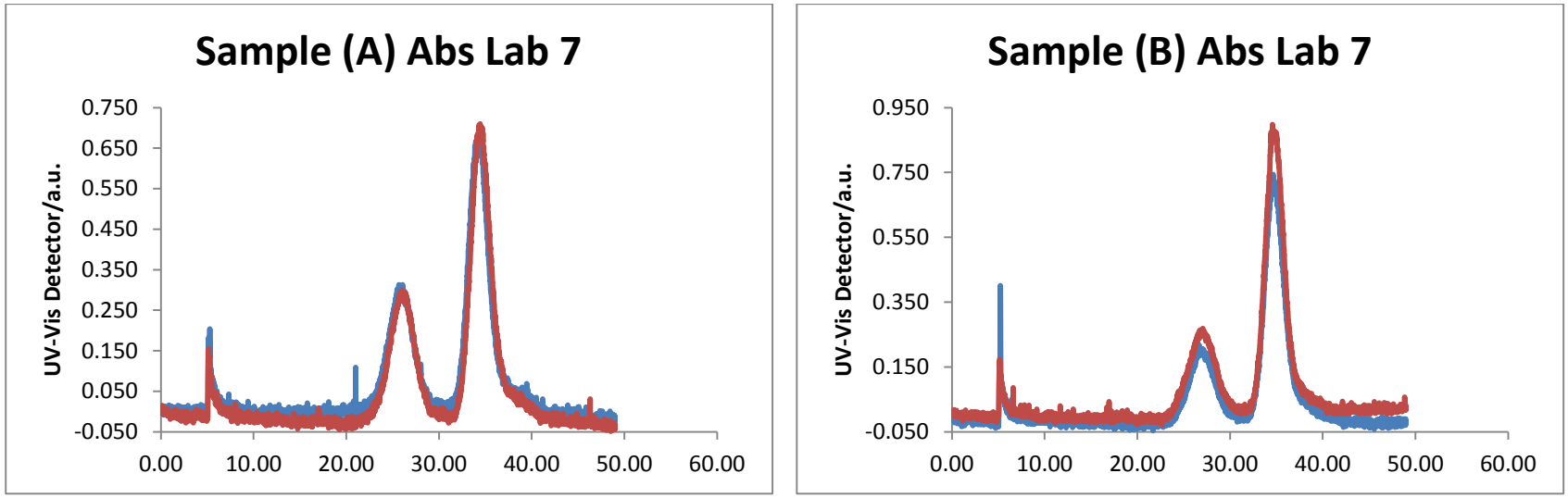
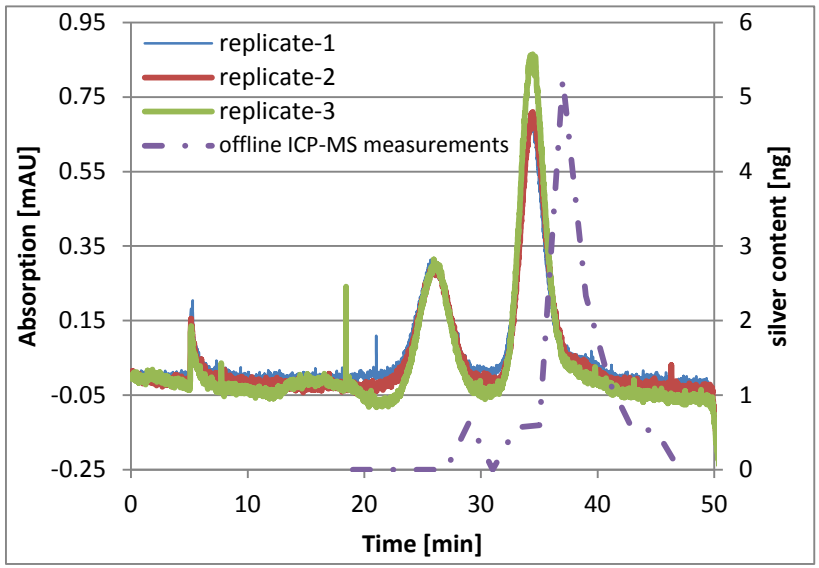
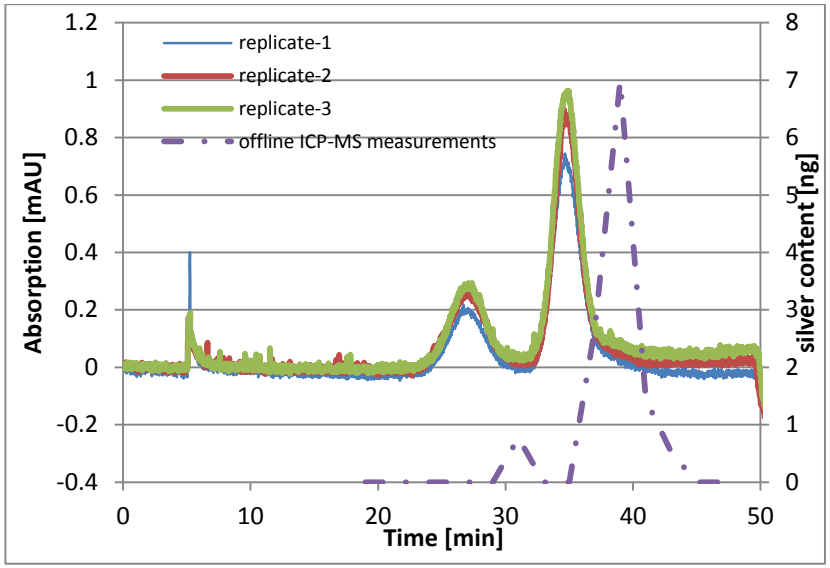


Figure 47 UV Results from Lab 7



Sample A



Sample B

Figure 48 UV and BATCH ICP-MS Results from Lab 7

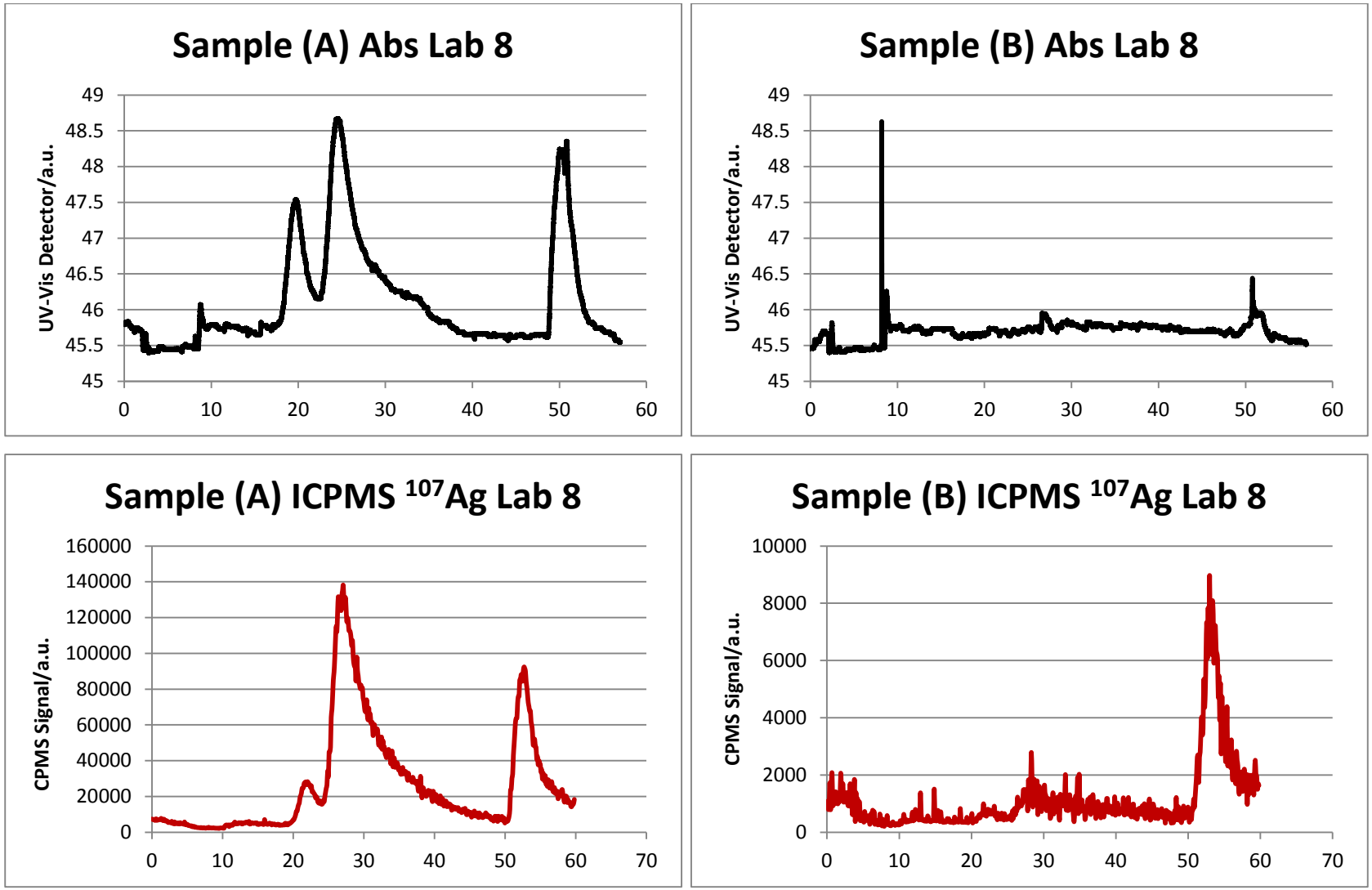


Figure 49 ICP-MS and UV Results from Lab 8

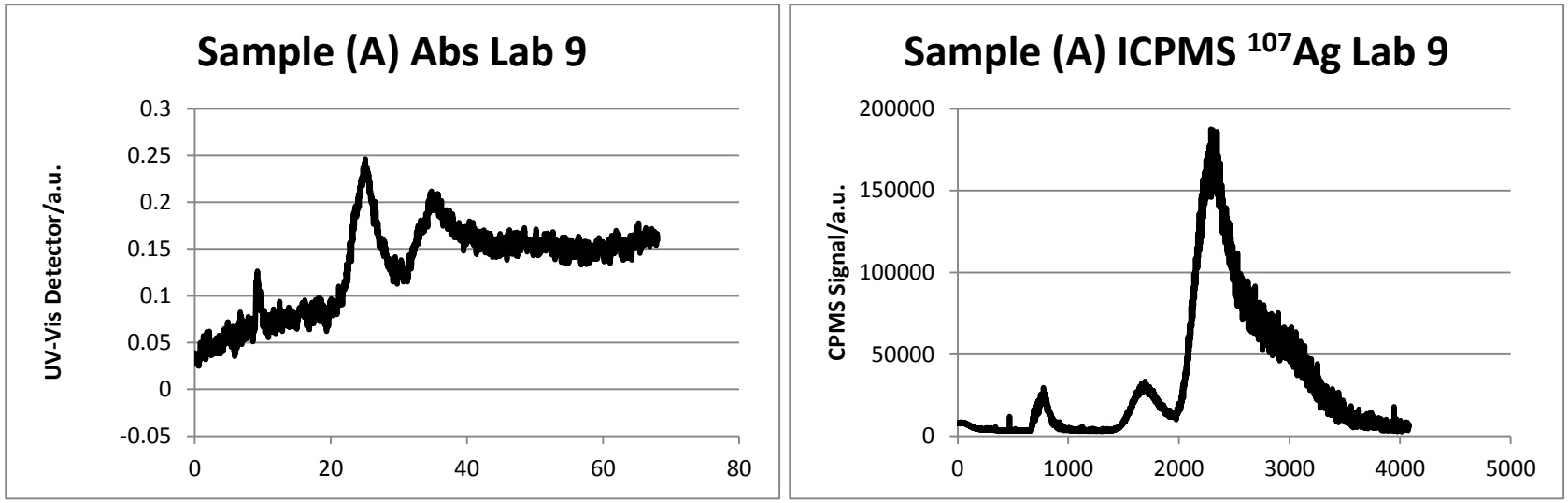


Figure 50 **ICP-MS and UV Results from Lab 9 Only sample A with replicate (Sample B not measured)**

Annex 3: Elution curves of trimodal mixture (20nm-40nm-80nm) using UV detection

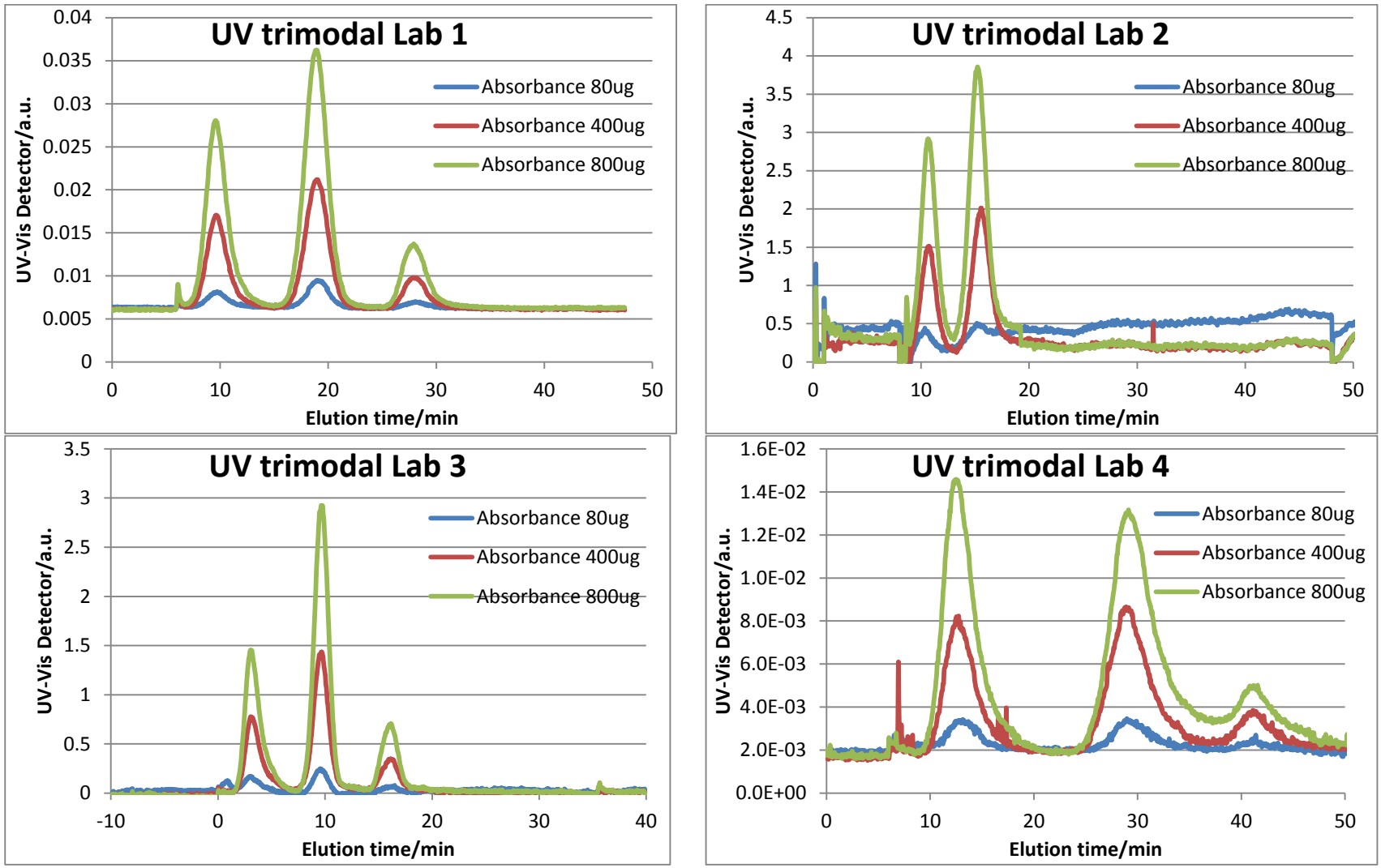
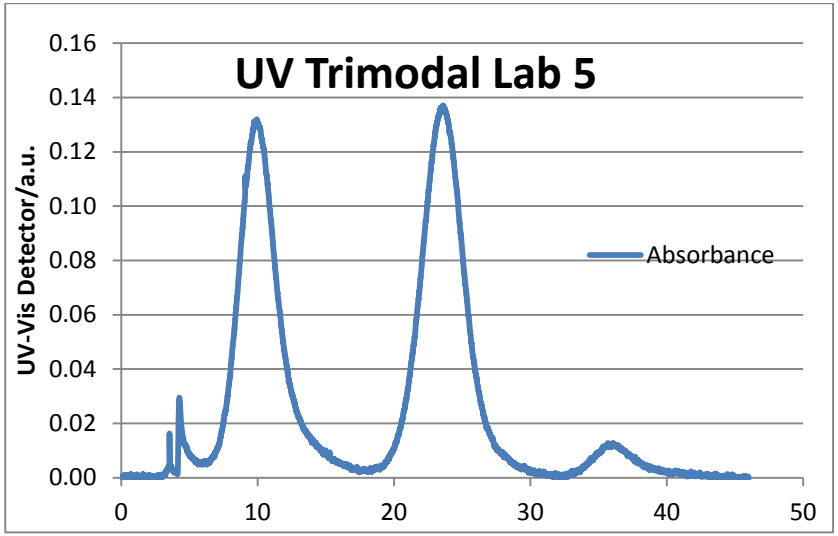
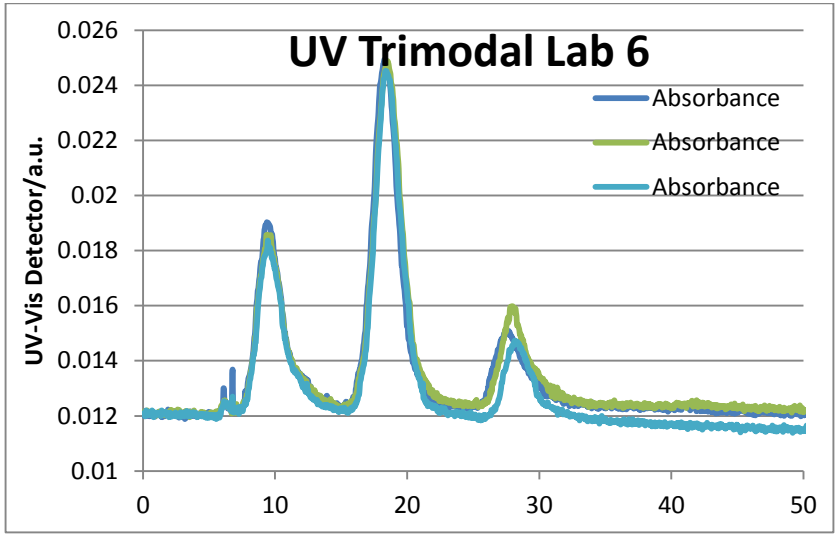
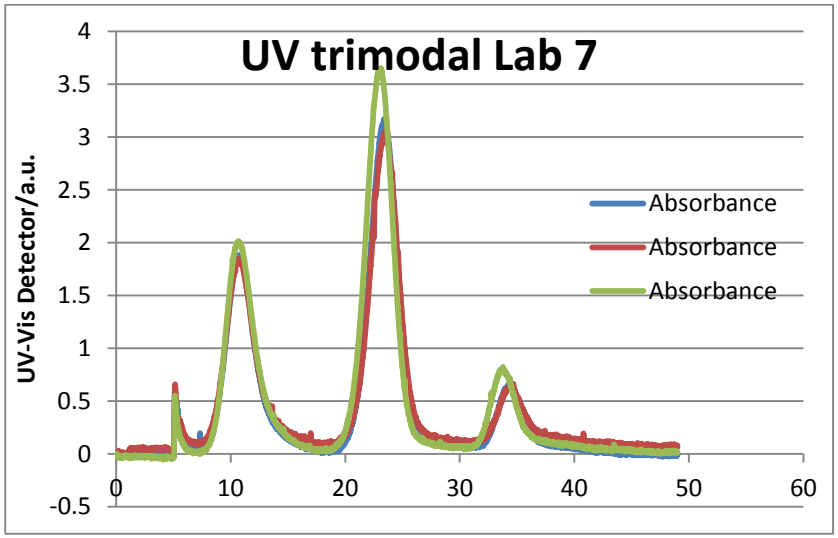


Figure 51 Labs 1-4Eelution curves of trimodal mixture







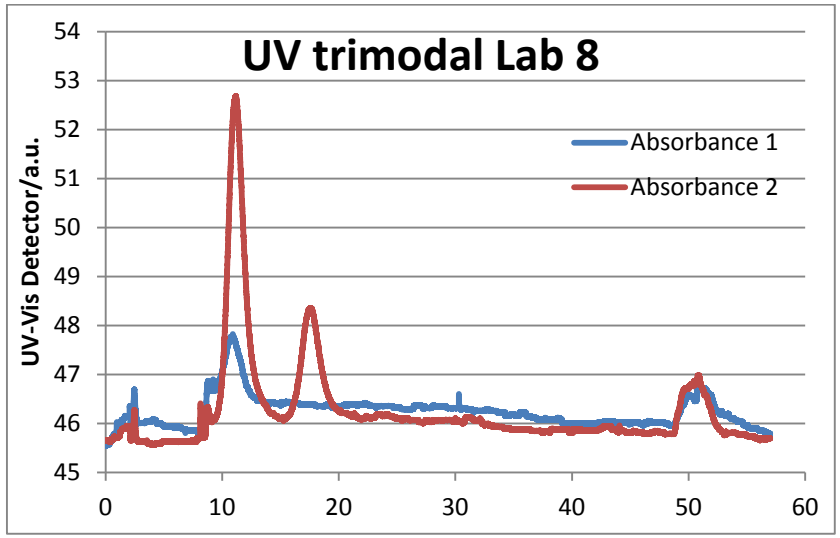


Figure 52 Labs 5-8 Elution curves of trimodal mixture

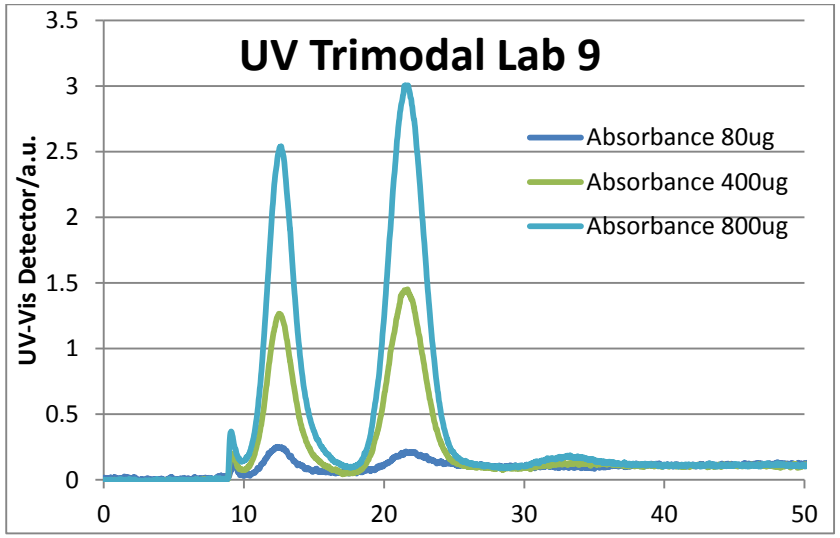


Figure 53 **Lab 9 E**lution curves of trimodal mixture

Annex 4: Summary of AF4-ICP-MS (off-line data) from laboratory 7

Participant Laboratory No.7 was not equipped with ICP-MS online but instead did sample fractionation with later analysis off-line. The samples for off-line analysis by ICP-MS were collected as 1ml fractions at a collection rate of 0.5ml/min. Analysis were conducted in a collaborator laboratory following the ICP-MS procedure described below. It should be noted that number of samples which could be analysed was limited and consequently not all runs could be fully evaluated nor could the repeatability be fully evaluated.

This method of analysing the nanoparticle size distribution was not foreseen in the current version of the SOP but the results obtained have been included in this report as they provide information on an alternative measurement strategy for laboratories where there is not the possibility of directly coupling of ICP-MS and AF4.

Description of procedure used to calibrate ICP-MS response to silver, sample preparation for offline ICP-MS measurements

- Direct calibration before offline measurements with: blank, 0.1, 0.2, 0.5, 1.0 and 2.0 µg/L Silver (in 1% Nitric acid, suprapur)
- Extern calibration with SRM 1643e, 1.036 µg/L Ag
- Intern standard: Rh, 30 µg/L
- Ionic silver standard solution (stock concentration 1g/L, supplier: Merck)
- Detection: ^{107}Ag, nogas-mode
- LOD for offline measurements: 0.05 µg/L (values < 0.05 µg/L are not given in the data evaluation)
- AF4-fractions: 1 mL → ICP-MS measurement value: mean of 2 minutes elution time
- Sample preparation: After AF4-run: Add 100 µl 65% nitric acid (suprapur), after at least 30 min diluting with ultrapure water → total sample volume = 10 mL → 1:10 dilution
- 10/40/100-mix: AF4 run on 30.4.2013, ICP-MS measurement on 2.5.2013
- 20/60-mix: AF4 run on 30.4.2013, ICP-MS measurement on 2.5.2013
- 40/60-mix: AF4 run on 30.4.2013, ICP-MS measurement on 2.5.2013 → fraction volume was too high to analyse two separated particle peaks (→ collecting smaller fractions would be better)
- Sample A: AF4 run on 6.5.2013, ICP-MS measurement on 17.5.2013
- Sample B: AF4 run on 6.5.2013, ICP-MS measurement on 17.5.2013

Additional Information and remarks:

- ICP-MS measurements could not be carried out immediately after the AF4 runs, time offset: between 3 and 11 days (the samples were stored in the dark at room temperature – no freezing)
- The sample preparation of AF4 fractions should be optimized in the future. The samples prepared for ICP-MS were not very stable (although the silver was dissolved in conc. nitric acid) → later measurements of the same sample gave different results (less silver content)
- Delay between AF4 signals (peaks) and ICP-MS signals (peaks) is because of the delay time between the AF4 detector and the fraction collector. The time offset of the ICP-analysis in the fraction can be clearly seen compared to the UV signal
- The analysis of the silver content of the unfractionated samples were in good agreement with the calculated silver concentration

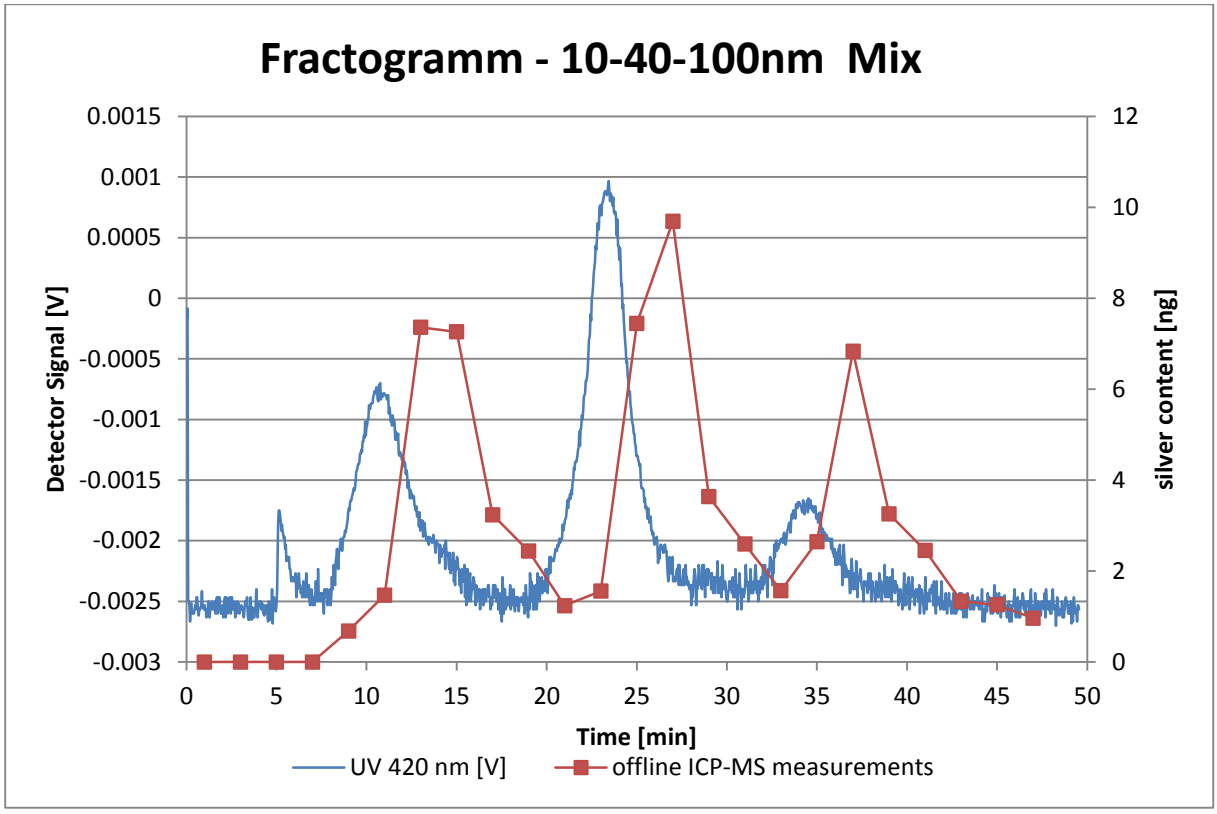


Figure 54 AF4-ICP_MS (off-line data) obtained from 10,40,100nm mixture

Table AA-1 Quantified AF4-ICP_MS (off-line data) obtained from 10-40-100nm mixture

	Measured/ng	Expected/ng	Recovery(%)
first peak	23.7	31.9	74.3
second peak	26.5	32.9	80.5
third peak	18.7	33.7	55.5
Total	68.9	98.5	69.9

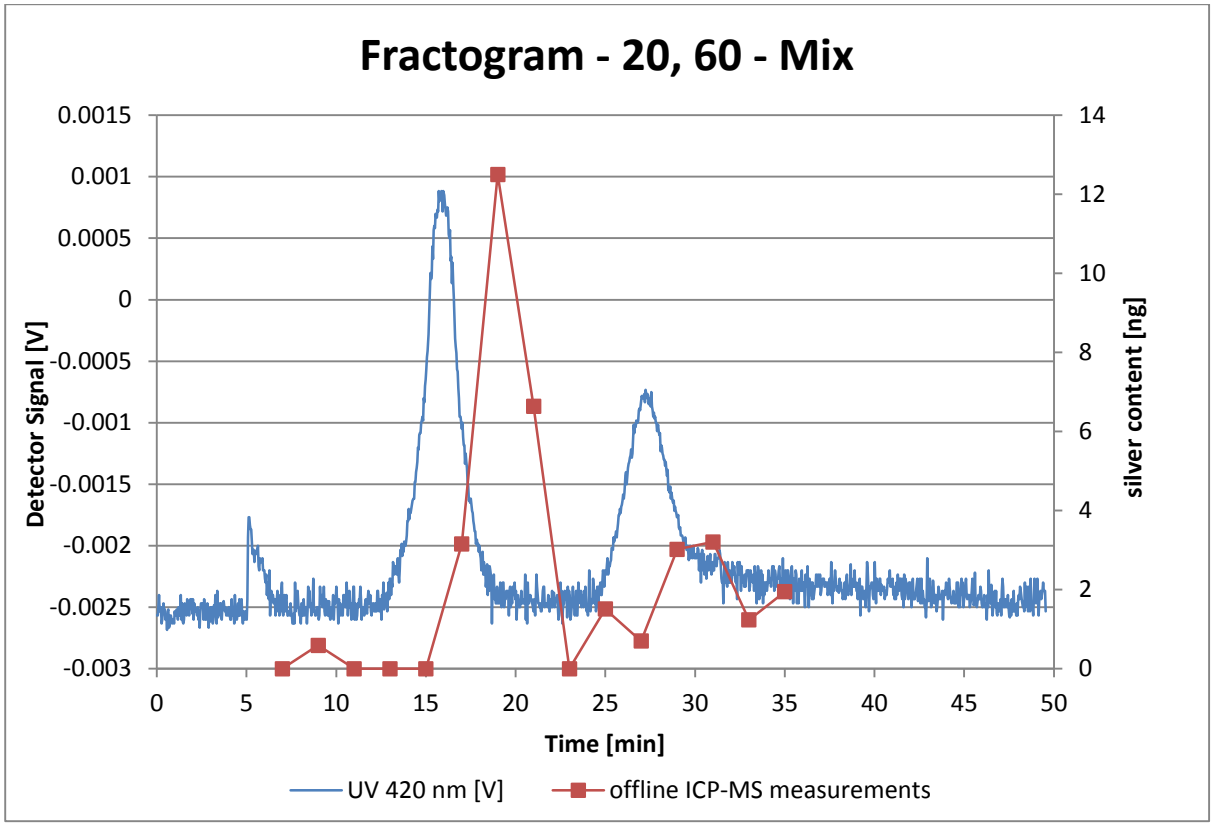


Figure 55 **AF4-ICP_MS (off-line data) obtained from 20-60nm mixture**

Table A4-2 Quantified AF4-ICP_MS (off-line data) obtained from 10,140,100nm mixture

	Measured/ng	Expected/ng	Recovery(%)
first peak	**22.9**	**34.4**	**66.6**
second peak	**11.6**	**34.9**	**33.2**
Total	**34.5**	**71.30**	**48.4**

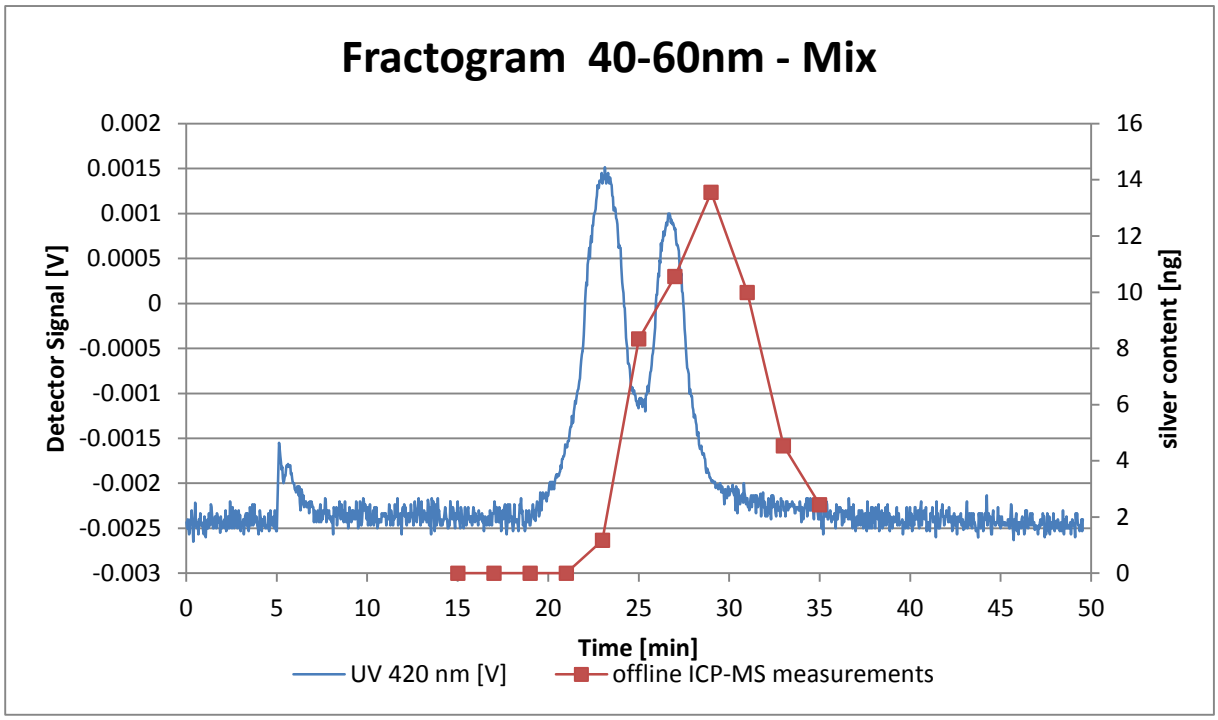


Figure 56 **AF4-ICP_MS (off-line data) obtained from 40, 60nm mixture**

Table A4-3 **Quantified AF4-ICP_MS (off-line data) obtained from 40-60nm mixture**

	Measured/ng	Expected/ng	Recovery(%)
first peak	**no peak separation**	**32.9**	**--**
second peak	**no peak separation**	**34.9**	
Total	**50.6**	**59.70**	**84.8**

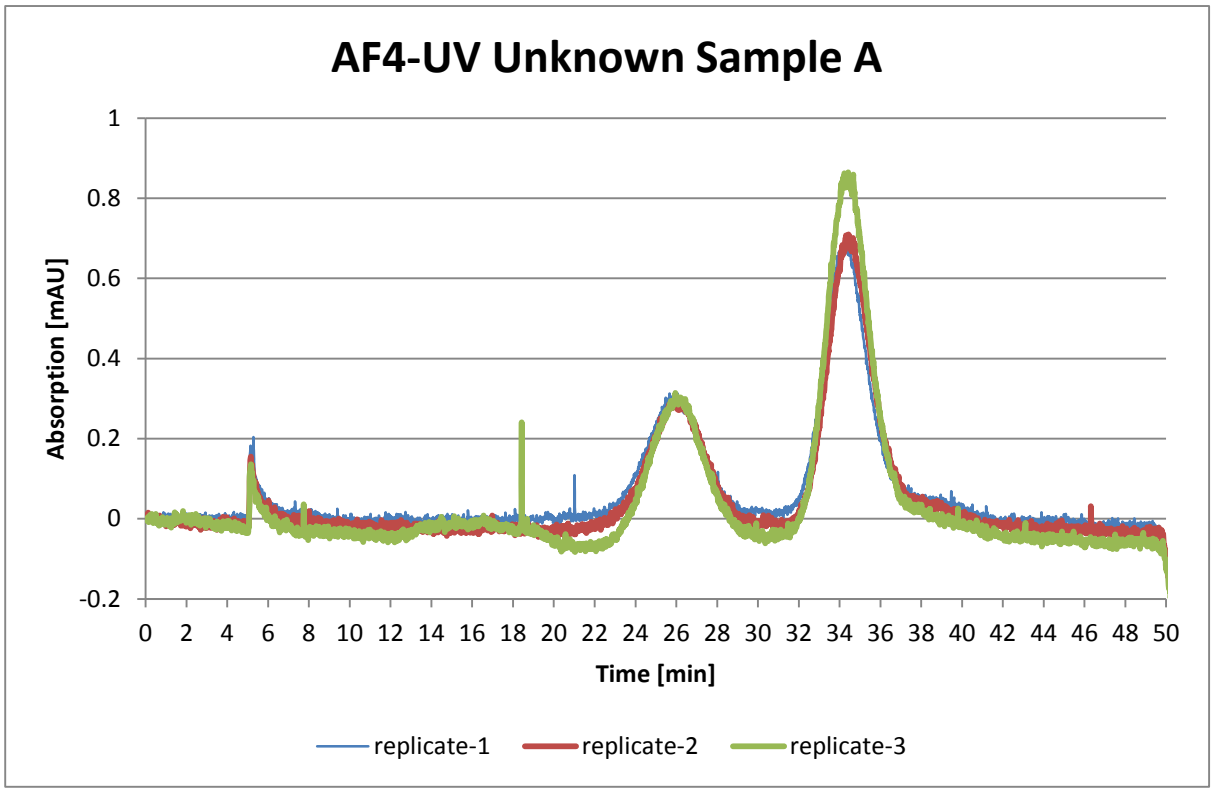


Figure 57 AF4-UV obtained from Unknown Sample A

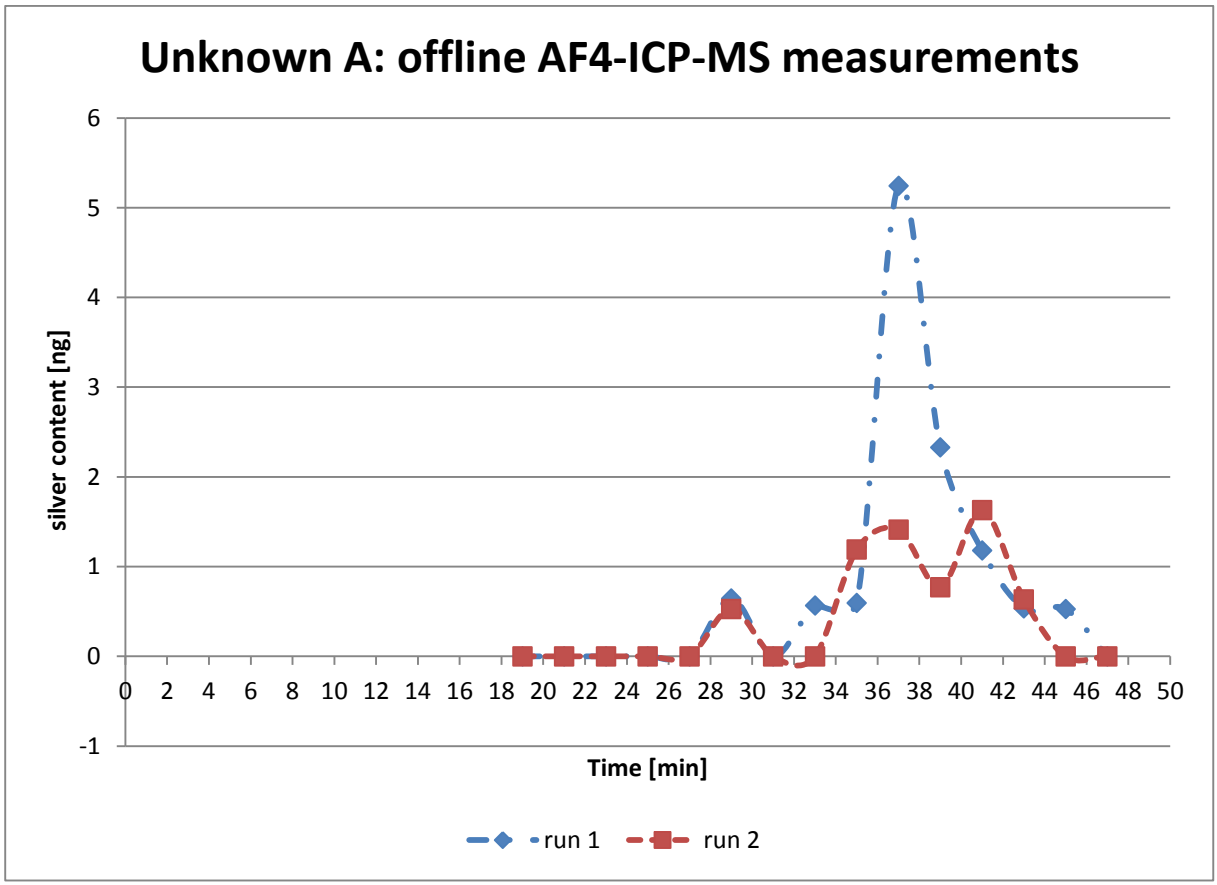


Figure 58 AF4-ICP-MS (off-line data) obtained from Unknown Sample A

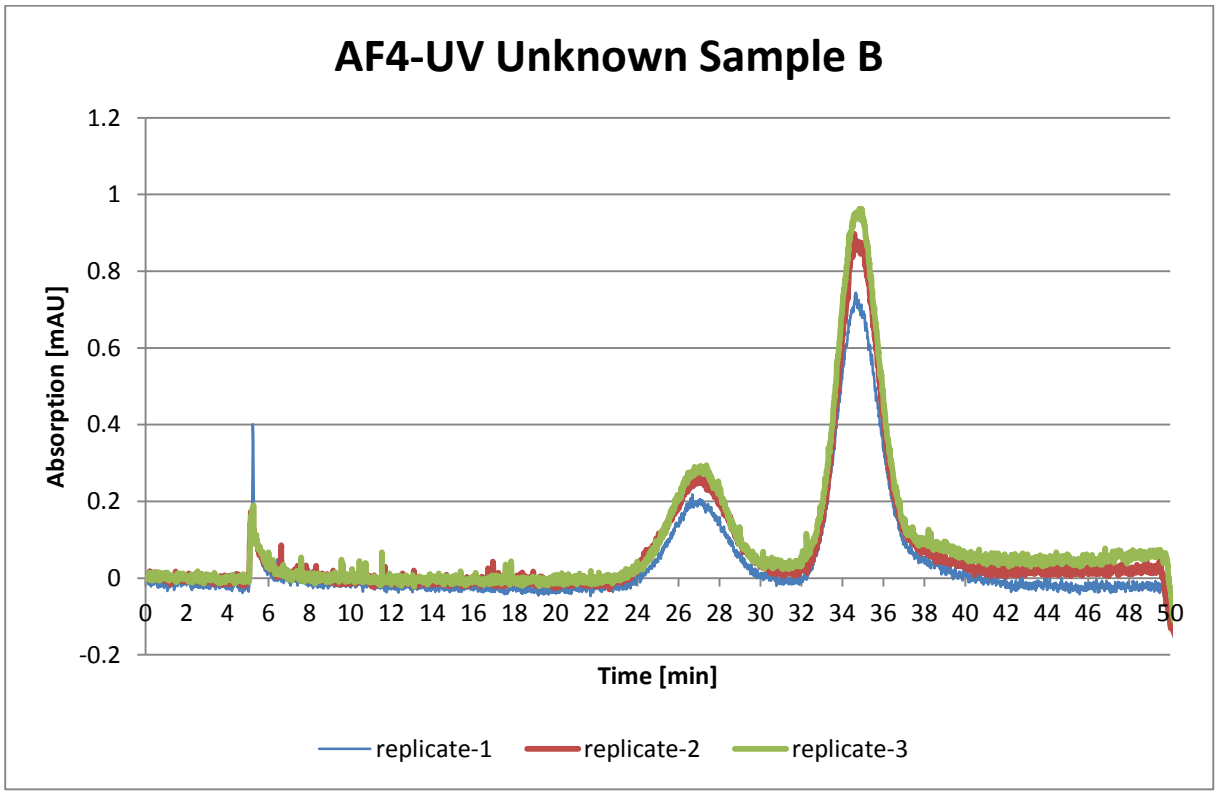


Figure 59 AF4-UV obtained from Unknown Sample B

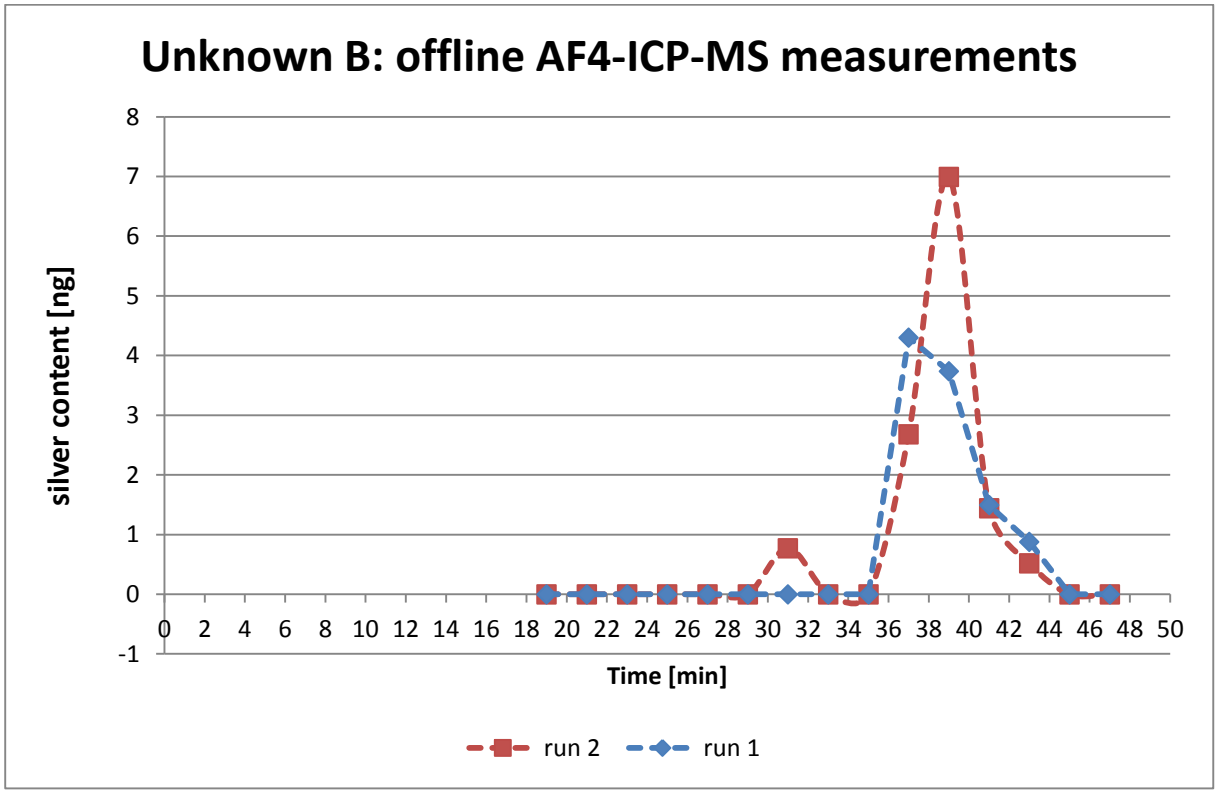


Figure 60 AF4-ICP-MS (off-line data) obtained from Unknown Sample B

Table A4-4 ICP-MS off-line results obtained for unknown samples A and B

		Measured/ng	Expected/ng*	Recovery(%)
Sample A Run 1	first peak	0.64	4.64	13.88
	second peak	10.98	35.91	30.57
	Total	11.62	40.55	28.66
Sample A Run 2	first peak	0.53	4.64	11.36
	second peak	5.64	35.91	15.69
	Total	6.16	40.55	15.20
Sample B Run 1	first peak	Not resolved	4.59	-
	second peak	10.40	35.78	29.08
	Total	10.40	40.37	25.77
Sample B Run 2	first peak	0.77	4.59	16.78
	second peak	11.63	35.78	32.51
	Total	12.40	40.37	30.72

*Expected values are calculated assuming an injected sample volume of 45µl and AgNP concentrations which correspond to those noted below (reproduced from the description of samples A and B given in section 8.1.)

Table A4-5 Mass concentrations of particles in unknown samples A and B

Unknown Sample	Conc. Particles of 50nm	Conc. Particles of 100nm
A	103 µg L^{-1}(4.64ng/45µl)	798 µg L^{-1} (35.91ng/45µl)
B	102 µg L^{-1}(4.59ng/45µl)	795 µg L^{-1}(35.78ng/45µl)

Considerations of the AF4-ICP-MS (off-line data)

The results obtained from the sample mixtures prepared from the undiluted mono-dispersed pseudo- standards show recovery values which are the range 55-80% and therefore consistent with levels of recovery routinely found with detection by UV alone. In particular the results obtained (**Figure** 54) with the tri-modal mix of 10,40 and 100nm particles show promise where the peak resolution required is more compatible with the eluent sampling frequency and volumes.

In contrast to this the ICP-MS results obtained with the unknown samples A and B are all below the 50% level which is lower than would have been expected. In considering these results a number of technical issues should be noted.

a) The samples A and B, as previously described were designed to simulate a bimodal particle distribution which is close to the nano/non-nano limit (50% particle number below 100nm) established by the EU definition. To simulate this ratio and maintain total concentration levels comparable with the method development exercises the mass concentration of particle of 50nm had to be reduced to a level which can be challenging to accurately quantify by ICP-MS.

b) In comparison to the sample mixtures made from the stock solutions, the samples A and B were subjected to the additional uncertainty coming from sample aging which appears to have been a greater issue than was foreseen.

c) The additional sample manipulation necessary for the completion of off-line analysis is likely to lead to higher loss of analyte material and in the particular case of Ag ions the mass loss through adsorption to contact materials (eg. vials/pipette tips) is known to be problematic.

The severity of each of these issues could be reduced by using more concentrated samples or by injection larger sample volumes. In this way relative errors due to mass loss would be reduced and size resolution could be increased by permitting higher sampling frequency.

Clearly the off-line methodology is unlikely to be able to compete directly with on-line measurements when using low concentration samples but as a strategy for use where direct coupling is not feasible these results suggest that the method is worthy of further study using more concentrated samples.

Annex 5: SOP document distributed to ring-trial participants.

Standard operating procedure for the inter-laboratory performance study: detection/quantification of silver nanoparticles in an aqueous matrix

Stage 1: Preliminary study to assess the correct application of the method in participating laboratories

Title:

Identification and quantification of Ag nanoparticles in aqueous suspensions combining Asymmetric Flow Field Flow Fractionation and Inductively Coupled Plasma-Mass Spectrometry (AF4-ICP-MS)

Version: Final Version for trial distribution

Contents

Annexes

1 Introduction

In 2011 the European Commission[1], in response to a request from the European parliament, agreed on a common definition for the term nanomaterial which requires that materials be characterized in terms of the number size distribution of their constituent particles. Implementation of this definition within the context of legislative controls requires that enforcement laboratories be provided with fit-for purpose analytical methods.

To address the analytical challenges and to provide valid technical input to potential legislators JRC-IHCP has, on specific request from DG-SANCO, began a study program aimed at the development of methods which can be applied to the technical implementation of the above noted definition. The outcome of this study ideally would result in the publication of a validated test protocol applicable to at least one technically relevant type of liquid dispersed nanoparticle.

As part of this activity a detailed study was undertaken by JRC to develop a specific method for the analysis of aqueous dispersed silver nanoparticles by use of combined Asymmetric Field Flow Fractionation (A4) and Induction Coupled Plasma-Mass Spectrometry (ICP-MS). This method has been now been developed into a simplified Standard Operating Procedure(SOP) which is detailed in this document. The further development of this procedure towards becoming a validated test protocol now requires the method be evaluated by inter-laboratory ring-trials.

Given the technical complexity of the problem, it has decided that the process of validating the SOP through inter-laboratory studies should be split in two stages: a method familiarization stage (Stage 1) and the validation study itself (Stage 2). This document will detail the procedures to be followed for participants in stage 1 of this process

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

The first stage of this study is to assess whether the analysis procedures developed by JRC can be successfully transferred to other independent laboratories equipped with comparable but not necessarily identical AF4-ICP-MS facilities. It is foreseen that the methodology be applicable by laboratories with existing experience of AF4 but not necessarily with experience of the nanoparticle mixtures of the type being examined here. It will, therefore, be necessary that each laboratory optimize the instrumental conditions for their own combination of equipment. To do this, a series of mono-modal (near mono-dispersed) samples of declared size and concentration are supplied. For an evaluation of the final optimized conditions simple bi/tri-modal samples of undeclared size and concentration are supplied for analysis. **The outcome of stage 1 will be discussed during a workshop shortly after its conclusion.**

Note [1] COMMISSION RECOMMENDATION on the definition of nanomaterial (2011/696/EU) published in the Official Journal of the European Union on 20.10.2011: L 275/38

2 Scope and applicability of the method and SOP

The procedure is applicable for the determination of electrostatically stabilized anionic silver nanoparticles with a particle size range 10-100 nm in ultrapure water. The method is based on on-line coupling of the particle size fractionation method asymmetric flow field flow fractionation with **I**nductively **C**oupled **P**lasma-**M**ass Spectrometry (AF4-ICP-MS).

3 Terms and definitions

AgNP	Silver nanoparticles
AgNP10, AgNP20…	Silver nanoparticles of nominal diameter of 10 nm, 20 nm…
AF4	Asymmetric Flow Field Flow Fractionation
UV/VIS	Ultraviolet/Visible
ICP-MS	Inductively coupled plasma mass spectrometry
ISTD	Internal standard
DG SANCO	Directorate General for Health & Consumers – European Commission
DG JRC	Directorate General Joint Research Centre – European Commission
SOP	Standard Operating Procedure

4 Principle of method

The untreated sample, injected in the sample loop of an AF4 system is subjected to size fraction separation by means of AF4 with smaller particles eluting before larger ones (range 10-100nm). For detection and quantification of the size fractions, the AF4 is coupled online to an ICP-MS.

When analysing unknown silver colloidal samples the size of nanoparticles is determined by calibrating the system for size against elution time using the supplied near mono-dispersed silver standards. For 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 following a method described later in section 7.5

5 Reagents

The study program foresees the use of a series of chemical reagents which will not be supplied by JRC but which should be available in each participating laboratory. During the analysis only chemicals of recognized high purity analytical grade should be used.

Water: Ultrapure (18 ohms resistivity)

Sodium hydroxide solution (NaOH): 0.1M in ultrapure water

Nitric acid, 67-69%: Ultrapure for trace analysis

Ionic silver standard solutions: Any commercially available certified ionic silver standard defined as being suitable for ICP-MS calibration

Internal standard for ICP-MS: Any commercially available certified ionic standard defined as being suitable for ICP-MS calibration may be used: Rhodium in nitric acid is recommended

Eluent of the AF4 system: Ultrapure water adjusted to pH 9.2 with diluted sodium hydroxide sodium hydroxide solution. The eluent shall be prepared freshly every day and shall be degassed before use.

Acidifier solution for AF4 post-column eluent (pre ICP-MS): 5% Nitric acid-may contain 20 µg L^{-1} Indium or Rhenium as a reference ion for dilution and flow monitoring.

Washing solution for ICP-MS: 2-5% Nitric acid solution

ICP-MS carrier gas: Argon (99,9999%)

Stock solutions of (mono-modal) silver nanoparticles: Supplied by JRC containing near mono-dispersed silver pseudo-standards[2] of citrate stabilized silver nanoparticles dispersed in aqueous sodium citrate solution. Nominal sizes range from 10nm to 100nm in 10nm steps. Concentrations and sizes are listed as follows in Table 1

[2] Pseudo standards refer to mono-dispersed nano-particle materials supplied for calibration in this study. Materials were obtained from two different commercial sources referred to as Manufacturer(1) and Manufacturer(2).

Table 1. Monodispersed silver nanoparticle pseudo-standards provided by JRC

Standard	Diameter TEM [nm]*	First Standard Deviation [nm]*	Hydrodynamic Diameter(DLS) [nm] *	Concentration by ICP-MS [μg mL^{-1}]***
AgNP10	10(±4)**	N/A	N/A	17.7
AgNP20	19.6	1.6	N/A	19.1
AgNP30	32.3	3.2	44.8	13.2
AgNP40	40.6	3.0	53.7	18.3
AgNP50	52.4	5.9	58.1	19.8
AgNP60	57.4	4.0	66.7	19.4
AgNP70	68.5	4.2	69.0	21.1
AgNP80	77.1	6.4	83.0	20.0
AgNP90	88.9	4.3	86.8	18.1
AgNP100	99.4	7.0	97.7	18.7

* Manufacturer(1) specification unless otherwise indicated

** Manufacturer(2) specification (8.6nm by Centrifugal Liquid Sedimentation)

*** JRC Laboratory (average concentrations on 3 replicate measurements)

6 Description of apparatus required for SOP

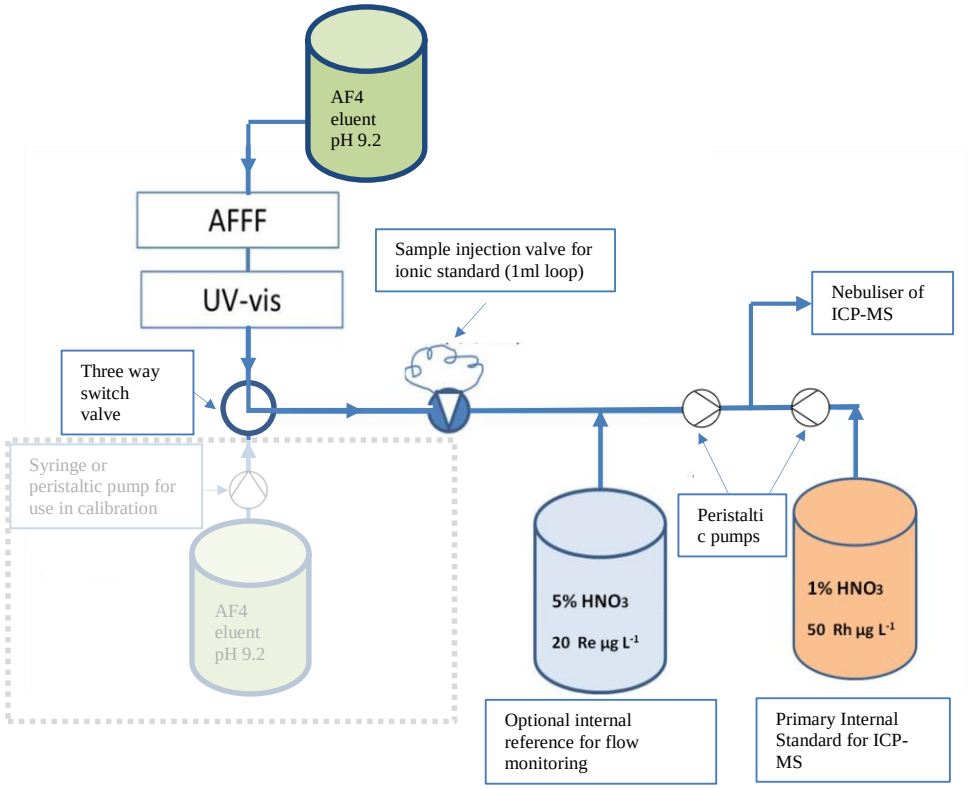
6.1 General apparatus

The method foresees the participating laboratories have access to the following apparatus:

- Suitable calibrated pipettes for sample dilution
- Ultrasonic bath for homogenization of particle solution
- pH meter for preparation of eluent solution
- Asymmetric flow field flow fractionation separator: Consisting of a least one solvent pump, solvent reservoir(s), a separation channel and a data acquisition and handling computer system.
- Inductively Coupled Plasma Mass Spectrometry (ICP-MS):

As can be seen from Fig.1 the elution output from the AF4 separation channel (or UV detector if present) is normally coupled directly (on-line) to the ICP-MS via a three-way switching valve. This valve is optional but allows the column to be rapidly isolated from the ICP-MS during ICP-MS mass calibration with ionic standards (section 7.5)

After leaving the separation channel and before entering the ICP-MS, the eluent should be acidified via a T-connector, using nitric acid solution (it may contain an additional internal 'dilution' standard such as Re at a concentration of 20 µg L^{-1}) and directed to the nebulizer of the ICP-MS for on-line detection and quantification of various AgNP size fractions. The mixing of the eluent with an acid solution is necessary to reduce possible loss of silver in the tubing before reaching the ICP-MS detector. The use of an internal reference for flow monitoring in the acidifier solution is optional but may be useful for monitoring dilution and AF4 outlet flow. On the contrary, an actual ISTD (i.e. Rh at a concentration of 50 µg L-1 in 1% nitric acid) to be used for Ag quantification, is



continuously added on-line using the pump of the ICP-MS nebulizer.

Fig.1 Schematic of preferred AF4-(UV)-ICP-MS online system

6.2 Specific information of apparatus used by JRC in method development

The following section will provide specific information on the equipment used by JRC in method development. *This data is provided for information only and participating laboratories are free to use AF4 conditions deemed most suitable to their own instrumentation and standard operating practices.*

6.2.1 AF4 equipment used by JRC in method development

The apparatus used in method development by JRC was a Postnova Model AF2000 equipped as follows. For alternative systems some of these parameters might change and require adaptation.

i. **Channel:** The channel used for the development of the current method was a Postnova patented channel with a channel length of 280mm and channel area=3160mm^2
ii. **Channel Spacer:** The channel spacer used for the development of the current method was 350 µm thick.
iii. **Channel Membrane:** The membrane material used for the development of the current method was regenerated cellulose (for aqueous applications) with a 10 kDa cut-off.
iv. **Injection Loop Volume:** The injection loop volume used for the development of the current method was 50 µL
v. **UV/Vis detector:** SPD-20AV from Postnova

6.2.2 ICP-MS equipment used by JRC in method development

JRC method development done using an Agilent 7700x ICP-MS system and the following conditions were set:

Table 2 Parameters used for ICP-MS instrument at JRC

Parameter	Details
RF-Power	1550 w
Reflected- Power	12.2 V
Temperature	ca. 6500-8000 K
Nebuliser type **Nebuliser flow rate**	MicroMist (quartz) 0.8 L min^{-1}
Spray Chamber	Scott (quartz)
Scan mode and Resolution	Time Resolved Analysis (TRA)
Integration Time	3.0 sec
Sampling Time	45 mins
Monitored masses	Ag(107, 109) Re(185, 187) Rh(103)

Connection between AF4 and ICP-MS in between runs was removed to allow a purge of the AF4 and a simultaneous cleaning of the ICP-MS with 2-5% nitric acid through the syringe pump. Background counts of Ag 107 were monitored till an acceptable and stable level was reached. Once AF4 and ICP-

MS were reconnected (with AF4 in pre-run conditions), 107, 103 and 187 counts were monitored and noted. New measurements were not started before acceptable ICP-MS conditions were reached (stable 187 and 103 counts and relatively low 107 background).

6.2.3 Use of UV-VIS Detector

Figure 1 shows a system equipped with a UV/VIS detector which is used as auxiliary detector for the preparatory steps of method optimization and later confirmation purposes with ICP-MS detection.
For the detection of silver nanoparticles across the size range 10-100 nm it is recommended that the UV/VIS detector monitoring wavelength is set at 420 nm which has been found to be a suitable compromise value which allows detection of all particles in the expected size-range.

7 Standard Operating Procedure(SOP)

7.1 Sample preparation and sample storage

Before using samples they must be brought to room temperature and homogenized for 1 minute with a vortex mixer prior to injection. If available it is additionally recommended to subject samples to a short 10s bath sonication.
All samples must be stored at 4°C away from extended exposure to light and air. Stock solutions of the pseudo-standard have been prepared under nitrogen and it is recommended that after opening, the head-space of the vial refilled with nitrogen before reclosing and returning to 4°C storage. Vials of undeclared mixtures should be opened only on the day of analysis.

7.2 Preparation of mixtures

Silver nanoparticle monodispersed standards of known size and concentration provided by JRC (Table 1) are mixed together by trial participants to obtain bimodal/trimodal mixtures.

7.2.1 AgNP10, AgNP40 and AgNP100 mixture *[approx. 800 ngmL^{-1} each]*

After thorough homogenization of standard solutions (sonication and vortexing), transfer exactly 40 µL of the 10 nm, the 40 nm and the 100 nm silver standard solutions into a vial and add exactly 880 µL of ultrapure water Mix (sonication and vortexing) thoroughly prior to injection.

7.2.2 AgNP20 and AgNP60 mixture [approx. 800 ng mL^{-1} each]

After through homogenization of standard solutions (sonication and vortexing), transfer exactly 40 µL of the 20nm and 40 µL of the 60nm silver standard solutions into a vial and add exactly 920 µL of ultrapure water. Mix (sonication and vortexing) thoroughly prior to injection.

7.2.3 AgNP40 and AgNP60 mixture [approx. 800 ng mL^{-1} each]

After through homogenization of standard solutions (sonication and vortexing) transfer exactly 40 µL of the 40 nm and 40 µL of the 60 nm silver standard solutions into a vial and add exactly 920 µL of ultrapure water. Mix (sonication and vortexing) thoroughly prior to injection.

7.3 Optimisation of elution parameters for particle recovery and separation

In this section the trial participants will be given the opportunity to familiarize themselves with some of the main separation parameters. If not already coupled to an ICP-MS, this part can be successfully completed using only a UV/VIS detector (wavelength set at 420 nm).

7.3.1 Optimisation of void peak size

One of the reasons for analyte loss in AF4 is a very pronounced void peak which in part may contain small particles which elute in an uncontrolled manner. The void peak size depends primarily on the injection/focusing time in relation to the injected volume and the initial cross-flow.

A too short injection/focusing time and/or a too low initial cross-flow, both lead to enlargement of the void peak. These conditions may be optimised in the following manner.

A series of injections for the optimization of the void peak size are done starting from a "standard" separation method/profile (see box below) using the mixture (***AgNP10, AgNP 40 and AgNP 100***) described in section 7.2.1

For users of PostNova systems the method/profile settings in the following box may be appropriate. For alternative equipment the choice of starting conditions must be determined from operator experience or from manufacturer recommended values.

Standard Separation Method Parameters for Postnova AF4: (see annex 2)

Focus Step

Injection Flow	:	0.2 mL min^{-1}
Injection time	:	5 mins
Cross-Flow	:	1 mL min^{-1}

Elution Step

Starting cross flow of 1 mL/min with linear decrease to 0.1mL min^{-1} during 40 min followed by a further constant cross flow of 0.1 mL min^{-1} for 10 min

Detector flow rate	:	0.5 mL min^{-1}
Injection sample loop volume	:	50 µL

The following proceed may be followed to optimize the conditions for acceptable void peak separation:

a) Using the tri-modal mixture (7.2.1) a series of elutions with different starting cross-flow values (increasing values in the range 0.5-2 mL min^{-1}) should be run to determine the minimum cross-flow conditions which permit full separation of the void peak and AgNP10 peak in the fractogram. In each of these elutions the values of injection flow and focus times must be kept constant. Suitable starting values for injection flow and time should be based on operator experience or instrument manufacturer recommendation for method development. To obtain a quantified measure of the void peak separation from the AgNP10 the capacity factor described in section 7.3.1.1 should be calculated. When separation conditions are found in which the capacity factor is >0.6 these values may be adopted for use in the next step of the optimisation.

b) Once the void peak can be fully separated from the AgNP10 peak various focusing times (injection times) shall be tried and the resulting void peak areas (sizes) compared with each other by overlaying the runs. An excessively short focusing time will produce a higher ratio of the void peak to the AgNP10 peak while excessively long focusing times may lead to distortion or loss of intensity of one or more of the AgNP10, AgNP 40 and AgNP 100 peaks. A compromise value of focusing time should be selected between these two extremes. The fractogram shown in Figure 2 illustrates an elution profile obtained with acceptable combination of cross-flow, focusing and injection times.

Results shall be recorded by providing overlaid fractograms of all runs and by filling the relevant table in Annex 3. Where possible, the data from the fractogram (Elution time and UV and ICP-MS isotopic signal as counts/second) should be made available in electronic format by inclusion in the appropriate excel template which will be supplied.

7.3.1.1 Determination of capacity factor (retention factor) k' for AgNP10

Solution to inject: The AgNP10, AgNP40 and AgNP100 mixture (prepared under 7.2.1) is injected.

The separation quality between the void and the AgNP10 peak is calculated from the fractogram by applying the following equation (see also example Fig 2).

$$k'(AgNP10) = (tR(AgNP10) - tV(void\ peak)) / tV\ (void\ peak)$$

where k'(AgNP10) is the capacity factor for AgNP10, tR(AgNP10) is the retention time of the AgNP10 peak and tV(void peak) is the retention time of the void peak.

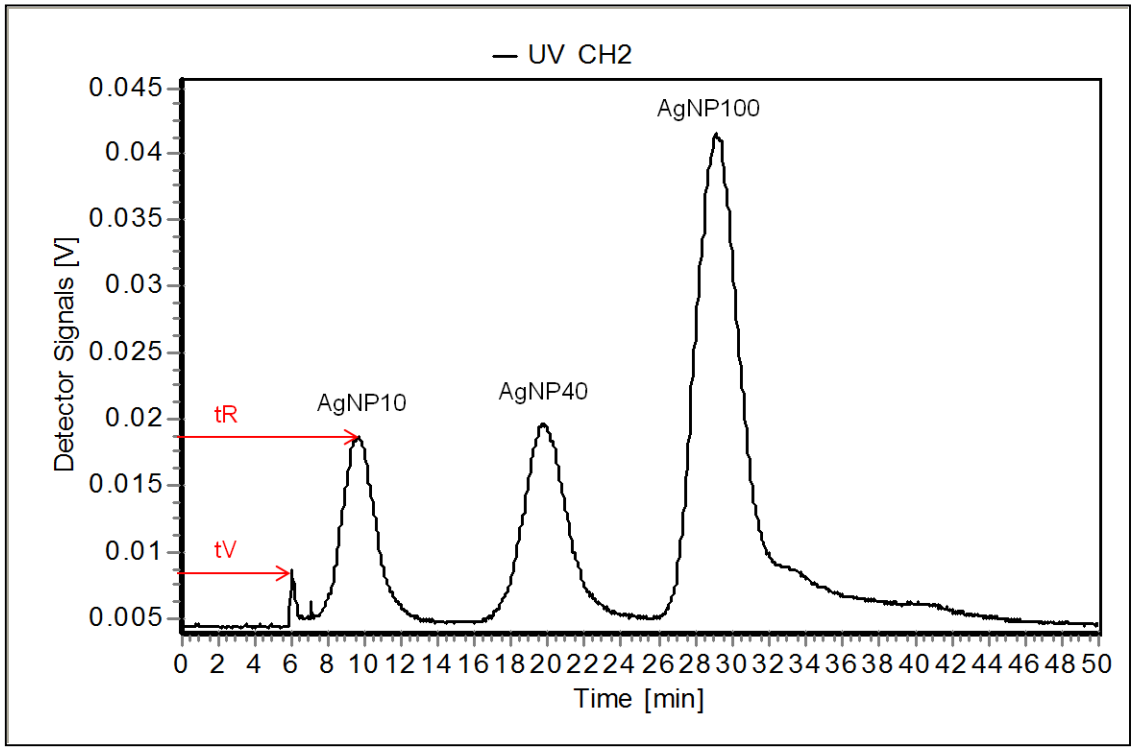


Fig 2. Fractogram example of an AgNP10/40/100 mixture (coupled to UV/VIS detector) ICP-MS detectors). In case it is smaller than 0.6, the AgNP10 fraction is eluted too quickly and will not be well separated from the void peak.

7.3.2 Optimization of peak separation parameters

Once suitable injection/focusing conditions have been determined as described in section 7.3.1 it is necessary to determine an elution step (eg. linear decrease from 1ml min^{-1} to 0.1ml min^{-1} in 40min) which allows an acceptable resolution between particles of different sizes. To assess the degree of separation achievable with any given elution step it will be necessary to calculate values of peak resolution using the method described in the following section 7.3.2.1.

7.3.2.1 Determining resolution between peaks

A measure of how well species have been separated is provided by measurement of the *resolution*. The resolution of two species, A and B, is defined as

$$R = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$

Where *W* is the peak width at the peak base and t_R is the retention time of peaks A and B.

Baseline resolution is achieved when $R = 1.5$

7.3.2.2 Optimisation of peak separation using tri-modal mixture (AgNP10/40/100)

This should be done by firstly analysing the trimodal mixture (AgNP10/40/100) and using the resulting fractogram to calculate the resolution between the AgNP10/40 and AgNP40/100 peaks as described previously. If the resolution achieved does not satisfy the minimum requisites quoted alternative elution conditions should be tested until the minimum required peak separation is achieved. The target should be to achieve a resolution as follows

a) for UV/VIS detector this should be at least R=1.5 for the fractions AgNP10/40 and R>1 for the fractions AgNP40/100.
b) for ICP-MS as detector this should be around 1 for both AgNP10/40 and AgNP 40/100 fractions. Coupling the AF4 to an ICP- MS leads to peak-broadening resulting in less optimized peak separations.

Results shall be recorded by providing the fractogram(s) and the calculated resolutions and recording them in Annex 3. Where possible, the data from the fractogram (Elution time and UV and ICP-MS isotopic signal as counts/second) should be made available in electronic format by inclusion in the appropriate excel template which will be supplied.

7.3.2.3 Optimisation of peak separation using (AgNP20 and AgNP60) mixture

Once the trimodal mixture can be satisfactorily separated the resulting optimised focusing/elution conditions should be used to measure the resolution using the biomodal mixtures AgNP20/60 prepared in sections 7.2.2. If the resolution achieved does not satisfy the minimum required values quoted below alternative elution conditions should be verified until the required peak separation is achieved.

The target should be to achieve a resolution of at least R=1.0 for the fractions AgNP20/60 (for both UV/VIS and ICP-MS detection)

Results shall be recorded by providing the fractogram(s) and the calculated resolutions and recording them in Annex 3

Where possible, the data from the fractogram (Elution time and UV and ICP-MS isotopic signal as counts/second) should be made available in electronic format by inclusion in the appropriate excel template which will be supplied.

7.3.2.4 Optimisation of peak separation using (AgNP40 and AgNP60) mixture

The final verification of the elution profile should be done using a sample of the AgNP40/60 mixture. The final step in optimisation requires that the elution of the AgNP40/60 mixture should show at least a separation of peak maxima as can be seen in Figure 3. With the optimised separation conditions it is probable that the AgNP40 and AgNP60 will not be base line separated. The aim is to achieve at least a separation of the peak maxima as shown in Figure 3.

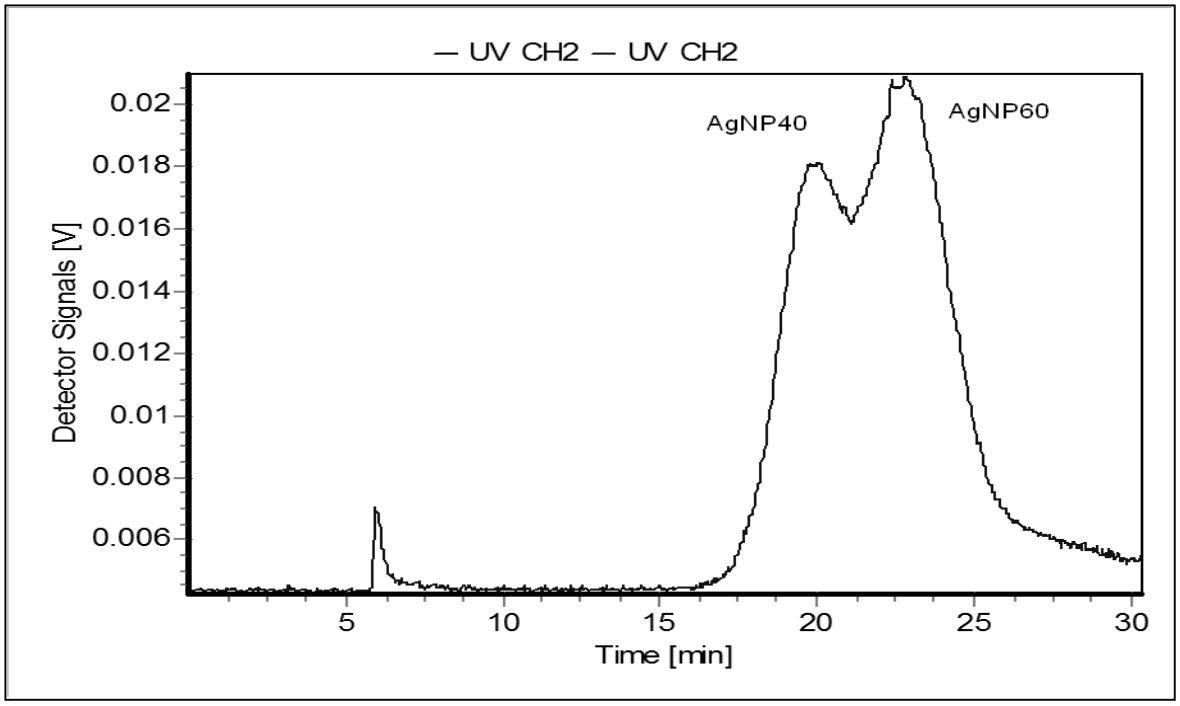


Fig 3. Separation of AgNP40 and AgNP60 fractions (coupled to UV/VIS detector)

If this level of separation is not observed then minor adjustments to the elution profile should be made until this is achieved.

Results shall be recorded by providing the fractogram(s) and where possible, the data from the fractogram (Elution time and UV and ICP-MS isotopic signal as counts/second) should be made available in electronic format by inclusion in the appropriate excel template which will be supplied.

7.4 Particle size determination

Retention times will be used as a means to determine particle size following a calibration curve obtained by injecting monomodal pseudo standards into the AF4 as follows.

7.4.1 Particle size calibration

The size of nanoparticles in the provided samples is determined by calibrating the AF4 for size against elution time using the supplied mono-dispersed silver standards (supplied by JRC).

All single mono-dispersed standards shall be diluted transferring 30 µL of the concentrated (provided) standard solution into a vial and adding subsequently 960 µL of ultrapure water resulting in approximate concentrations of 600 ng mL^{-1}. For exact concentrations please calculate using concentrations listed in Table 1.

These diluted fractions are injected singularly (or in peak-maxima separated mixtures) and the resulting retention times plotted against the particle diameter (see example Figure 4).

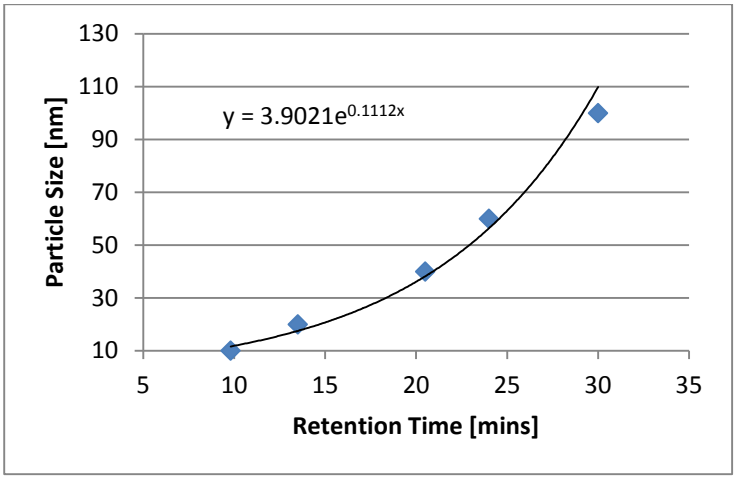


Fig 4. Example of dependency of particle size on retention time

An interpolated data points fitting curve (trendline, see example in Figure 4) can be easily extracted with most spreadsheet software packages (e.g. MS Excel, Origin).

Results of the size-retention time calibration shall be recorded in the excel template supplied.

7.5 Quantification of silver in identified particle size fractions

Silver concentration of size fractions (peaks) in sample mixture may be determined as usually done in participants' laboratory. In alternative to this may be done by calibration post-channel with ionic silver solutions (not provided by JRC) as described below in section 7.5.1. In either case it is requested that participants include a description of the method used in reporting Annex 4

7.5.1 Post-column calibration with ionic silver solution

All the following steps have to be performed daily, once ICP-MS conditions have been monitored, tuning performed and usual quality requirements fulfilled. Once the ICP-MS is running in chromatographic (or time resolved) mode according to section 6, the post column calibration approach can be used.

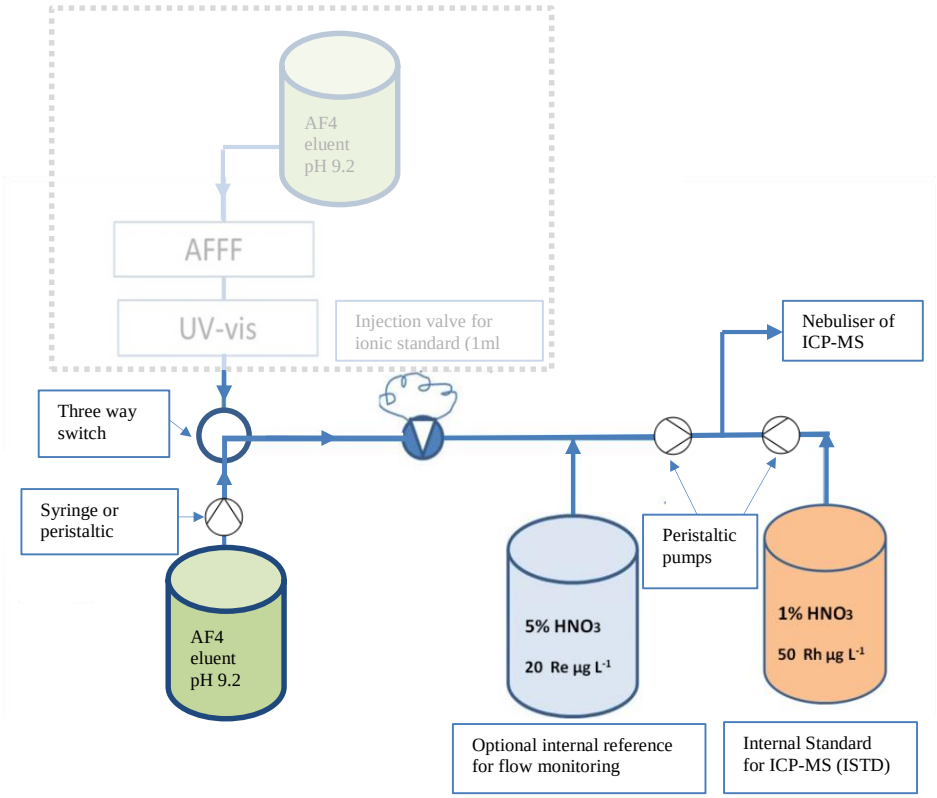


Fig 5. Possible set-up for ionic post-column calibration (AF4 Column excluded)

In this approach, the AF4 outlet is disconnected from the ICP-MS detector using the three switching valve and a peristaltic or syringe pump is used instead, simulating the flow of the AF4 while reducing the silver background signal from the column (see fig. 5). The flow of this pump must be set at the same flow rate as the AF4 outlet (0.5ml/min) and it has to pump freshly prepared AF4 eluent set at pH 9.2. The ionic standards have to be diluted in freshly prepared AF4 eluent set at pH 9.2 at exact concentration using a range that is suitable for expected level of silver in the fractions eluting from the AF4. With the JRC instrument a 5 point calibration of 0, 1, 12.5, 25, 50 ng mL^{-1} in AF4 eluent was normally done.

The ICP-MS is calibrated post-channel by injecting ionic silver calibration solutions through a sample introduction valve equipped with a 1 mL loop. During the entire process both dilution and internal standards have to be in place simulating the set-up in place during a normal AF4-ICP-MS run. Signals to be monitored are 107, 109, 103, 185 and 187 as described before.

For quantification, silver signal (107) has to be divided by ISTD signal (i.e. 103 for Rh) for both samples and calibrants. Ratio 107/103 has to be plotted *versus* retention time on the ICP-MS detector (in minutes). Peak area can be integrated by using available software or statistic environments. Finally areas have to be used versus concentrations to create calibration curves. A simple linear regression function can be used, without forcing it through the origin. Ionic calibration has to be run at least once a day and every time the plasma is switched on/off and tuned.

7.6 Evaluation of ICP-MS detector response to particle size

In this test it is requested that the previously used tri-modal mixture of nanoparticles be analysed at three different total concentrations. This section serves to verify whether the ICP-MS response to the eluted Ag nanoparticles can be assumed to be independent of particle size.

7.6.1 Sample Preparation

1) Sample preparation *(Tri-modal mix 800*ng mL^{-1}*)*: A fresh stock solution of AgNP10, AgNP40 and AgNP100 mixture should be prepared as previously detailed in section 7.2.1: *[approx. 800ng* mL^{-1} *of each size)*

2) 50% tri-modal sample (*Tri-modal mix 400*ng mL^{-1}*)*: 500ul of the above tri-modal mix should be diluted in 500ul of ultrapure water to give a solution containing approx. 400ng mL^{-1} of each size)

3) 10% tri-modal sample (*Tri-modal mix 80*ng mL^{-1}*)*: 100ul of the stock solution *(Tri-modal mix 800*ng mL^{-1}*)* should be diluted in 900ul of ultrapure water to give a solution approx. 80ng mL^{-1} of each size.

7.6.2 Mass calibration of ICP-MS

The ICP-MS system should be calibrated as normally done in the participant's laboratory or alternatively using the ionic standards as described in section 7.5.

7.6.3 Analysis of tri-modal mixtures

Using the optimized elution profile previously developed the three samples of the tri-modal stock solution, (80ng mL^{-1}, 400ng mL^{-1} and 800ng mL^{-1}), should be analysed in order of increasing concentration.

Results shall be recorded in Annex 4 by providing the fractogram graphs and the calculated concentrations for each of the three particle sizes. Where possible, the data from the fractogram (Elution time and UV and ICP-MS isotopic signal as counts/second) should be made available in electronic format by inclusion in the appropriate excel template which will be supplied.

7.7 Determination of particle fraction sizes and concentrations in <u>unknown samples</u>

To assess the effectiveness of the methodology in an impartial manner, participants will be required to analyse a series of two samples each of which contains at a mixture of at least two size(s) of silver nanoparticle (size range 10-100 nm) in a concentration range between 100-1000ng mL^{-1}. The samples will be labelled UNKNOWN SAMPLE A, and UNKNOWN SAMPLE B and no information will be provide about particle sizes or concentrations

7.7.1 Analysis of unknown sample A

The unknown sample A should be diluted before use. After thorough homogenization of the solution (sonication and vortexing), transfer exactly 50 µL of the mix into a vial and add exactly 950 µL of ultrapure water. Mix (sonication and vortexing) thoroughly prior to injection.

The diameters of the silver nanoparticles contained in the sample are calculated from the curve of elution time-particle size determined in 7.4.

The concentration of the eluted silver fractions is determined as described under 7.5 Results are recorded in Annex 5. Where possible, the data from the fractogram (Elution time and UV and ICP-MS isotopic signal as counts/second) should be made available in electronic format by inclusion in the appropriate excel template which will be supplied.

7.7.2 Analysis of unknown sample B

The unknown sample B should be analysed as received (no dilution necessary). The homogenized (bath sonicated and vortexed) sample should be injected into the AF4-ICP-MS system and eluted according to the optimise procedure determined previously in section 7.3

The diameters of the silver nanoparticles contained in the sample are calculated from the data fitting curve as determined in 7.4.

The concentration of the eluted silver fractions is determined as described under 7.6 Results are recorded in Annex 5. Where possible, the data from the fractogram (Elution time and UV and ICP-MS isotopic signal as counts/second) should be made available in electronic format by inclusion in the appropriate excel template which will be supplied.

8 Reporting of results

All results shall be reported in the tables found in annexes 3, 4 and 5. Relevant fractograms and calibration curves shall be provided as supporting materials in Word or PDF format. Where possible the data from the fractograms (Elution time, UV and ICP-MS ion signals) should be made available in an electronic format in the excel files which will be supplied.

Please return annexes by e-mail to Douglas Gilliland at the following address:

Email: douglas.gilliland@jrc.ec.europa.eu

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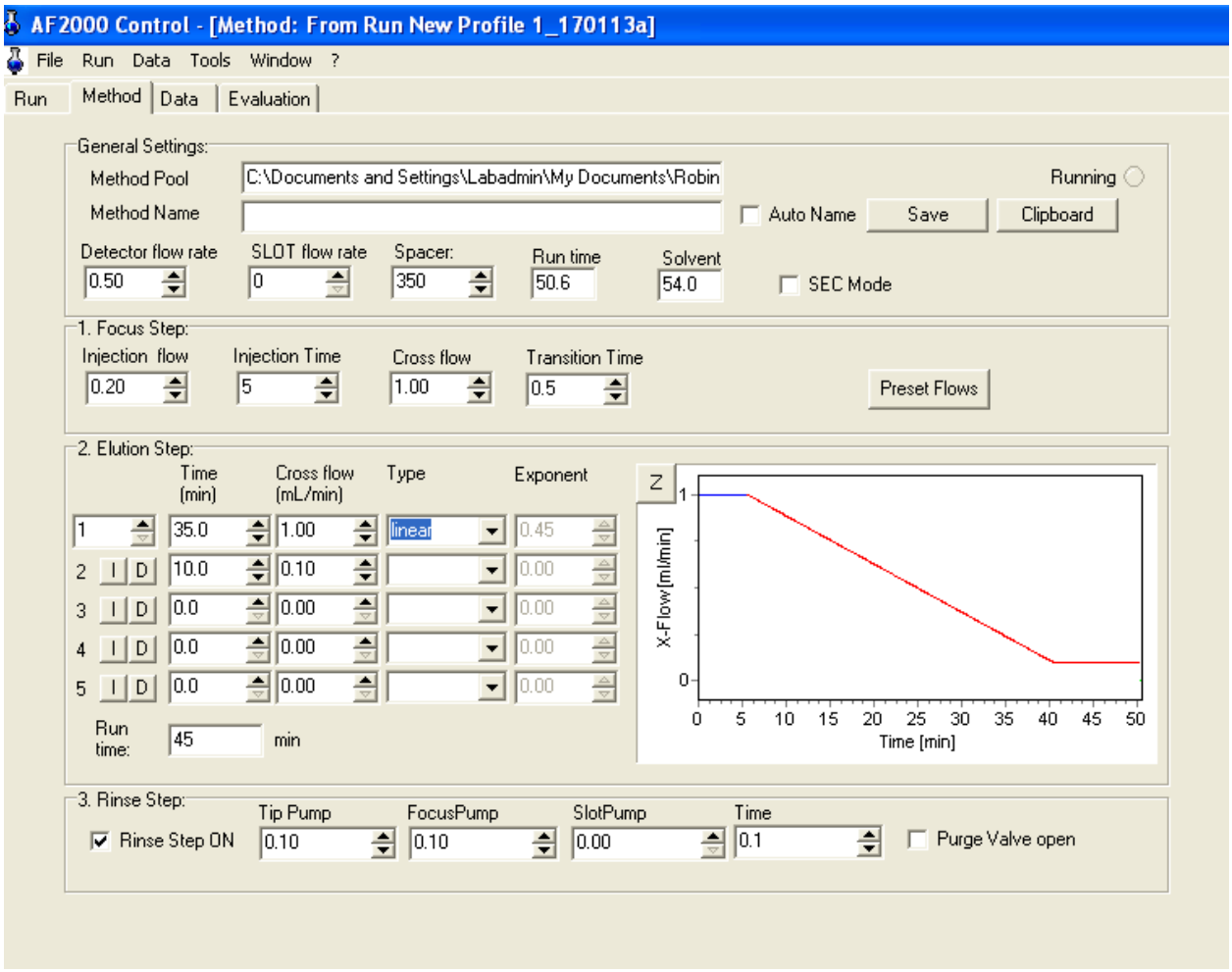
Annex 1: List of materials provided by JRC

The parcel send out by JRC contains the following items:

- Vial with AgNP10 stock-solution
- Vial with AgNP20 stock-solution
- Vial with AgNP30 stock-solution
- Vial with AgNP40 stock-solution
- Vial with AgNP50 stock-solution
- Vial with AgNP60 stock-solution
- Vial with AgNP70 stock-solution
- Vial with AgNP80 stock-solution
- Vial with AgNP90 stock-solution
- Vial with AgNP100 stock-solution
- Vial with unknown Sample A (requires dilution)
- Vial with unknown Sample B(to be used as supplied)
- Materials Safety Data Sheet

Annex 2: Parameters for AF4 elution method developed on Postnova AF2000



Annex 3: Result Sheet: Optimisation of Separation Parameters (Section 7.3)

A.3.1 General Information

Name of Company/ Research Institution	
Address	
Contact Person	
Email Contact Person	
Phone Contact Person	
Period when analysis were done (date)	

A.3.2 Instrumental Information

A4 Specifications (Manufacturer, Model)	
AF4 Loop Volume [µL]	
Separation Channel Specifications (Size)	
Membrane used (Type, Part#, Lot#)	
ICP-MS specifications (Manufacturer, Model, spray chamber, nebulizer, cones, nebulizer pump flow)	

A.3.3 Optimisation of void peak size [see section 7.3.1]

A.3.3.1 Optimisation of void peak size – Results during development

Attempts	Injection Loop Volume [mL]	Initial Cross-Flow [mL/min]	Injection Flow [mL/min]	Injection time [min]	Void Peak Area [AU]
1					
2					
3					
4					
5					

A3.3.2 Final optimised parameters

Parameters	Values
Injection Flow [mL/min]	
Injection Time [min]	
Initial Cross-Flow [mL/min]	

Please annex all relevant fractograms

A3.3.3 Capacity factor under optimised conditions [section 7.3.1)

Date of Analysis: . .

Capacity factor k' for AgNP10

k'(AgNP10)	

Please provide fractograms as supporting material.

A.3.4 Optimisation of peak separation– results under optimised conditions (section 7.3.2)

A.3.4.1 Resolution calculation for AgNP10/40 and AgNP40/100 (section 7.3.2.1)

R(AgNP 10/40)	
R(AgNP 40/100)	

Please provide fractograms as supporting material.

A.3.4.2 Resolution calculation for AgNP20/60 (section 7.3.2.2)

R(AgNP 20/60)	

Please provide fractograms as supporting material.

A.3.4.3 Verification of peak separation using (AgNP40 and AgNP60) mixture (section 7.3.2.3)

FRACTOGRAM OF (AgNP40/AgNP60) MIXTURE (Section 7.3.2.3)

Please provide fractograms as supporting material.

Annex 4: Result of known Tri-modal sample mixtures (approx. 80, 400 and 800 ng mL^{-1})

A.4.1 Trimodal mixture approx. 80 ng mL^{-1} Date of Analysis: .

Detected size fractions [nm (e.g. 10nm, 40nm, 80nm)		Ag mass in each peak fraction	
Fraction	Particle Size [Diameter, nm]	Measured mass of Ag	Expected mass of Ag(1)
1	10nm		
2	40nm		
3	100nm		

Please provide fractograms as supporting material.

A.4.2 Trimodal mixture approx. 400 ng mL^{-1} Date of Analysis: .

Detected size fractions [nm (e.g. 10nm, 40nm, 80nm)		Ag mass in each peak fraction	
Fraction	Particle Size [Diameter, nm]	Measured mass of Ag	Expected mass of Ag(1)
1	10nm		
2	40nm		
3	100nm		

Please provide fractograms as supporting material.

A.4.3 Trimodal mixture approx. 800 ng mL^{-1} Date of Analysis: .

Detected size fractions [nm (e.g. 10nm, 40nm, 80nm)		Ag mass in each peak fraction	
Fraction	Particle Size [Diameter, nm]	Measured mass of Ag	Expected mass of Ag(1)
1	10nm		
2	40nm		
3	100nm		

Please provide fractograms as supporting material.

(1) Values of expected concentrations should be calculated from actual dilutions done (if different from those in the section 7.6.1) and assuming the Ag concentrations for each mono-modal stock solution as detailed in Table 1

A4.4.4 Description of procedure used to calibrate ICP-MS response to silver

Annex 5: Result of unknown samples (A) and (B)

A.5.1 Sample ID: UNKNOWN SAMPLE A **Date of Analysis:** . .

Detected size fractions [nm] (e.g. 10nm, 40nm, 80nm)		
Fraction	Particle Size [Diameter, nm]	Concentration [ng mL^{-1}]
1*		
2		
3		
4		

* numbering is arbitrary and does not mean that this sample contains 4 different particle size fractions

Please provide fractograms as supporting material.

Fractogram of Unknown mixture A (diluted)

FRACTOGRAM OF UNKNOWN MIXTURE A (DILUTED) (Section 7.7.1)

A.5.2 Sample ID: UNKNOWN SAMPLE B Date of Analysis: .

Detected size fractions [nm] (e.g. 10nm, 40nm, 80nm)		
Fraction	Particle Size [Diameter, nm]	Concentration [ng mL^{-1}]
1*		
2		
3		
4		

* numbering is arbitrary and does not mean that this sample contains 4 different particle size fractions

Please provide fractograms as supporting material.

Fractogram of unknown sample B (as supplied)

FRACTOGRAM OF UNKNOWN MIXTURE A (as supplied) (Section 7.7.2)

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