Physicochemical characterisation of silica nanoparticles in complex food matrices

12CMP056 Project Report (Placement at LGC)

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1. Abstract

Manufactured nanoparticles incorporation into food has offered many advantages to the food industries as well as for the consumers. There are currently a variety of consumer products that contain nanomaterial. Due to the availability of the products there has been increasing use and consumption of food containing nano-sized particles. Concerns have risen particularly on whether consumption of nano-sized particles could potentially be toxic to humans. In order to assess the potential toxic effects, characterisation of nanoparticles must be performed. This is important because assessment of toxicity would be not possible until a set of nanomaterials with defined physico-chemical properties are available. For example nanomaterials:

- composition
- morphology (size and shape)
- surface area
- surface layer
- surface charge
- solubility

At the moment no single technique can provide all of the above information about the nanoparticle. As a result currently there are no standardised protocols available for the chacterisation of nanoparticle in complex matrices, thus a range of analytical techniques will be required. Characterisation of nanoparticles will enable international regulators for example (BSI) NTI/1, UK committee for the standardisation of nanotechnologies to set out new proposals for ISO standards. These include for example guidelines on the certification of consumer products to give a list of approved nanoparticles, to ensure the safety of consumer products before begin marketed. In addition regulators and industries are working towards labelling of nano consumer products that contains information about the nanoparticles for example its size range, concentration etc.

The experimental work aims to compare several methods developed earlier by LGC for the characterisation of silica nanoparticles present in coffee creamer sample complex matrices. Coffee creamers are known to contain synthetic amorphous silica (SiO_2) and have been used for many years. It is also known as a food addictive (E551), thou it is permitted for application in food, there is still a lack of information concerning the safety of consuming

(E551) nanosilica. Studies on the (FFF) parameters were carried out as part of the training, based on earlier work by (LGC). This involved investigating method optimisation of (FFF) parameters, based on earlier work by developed LGC. Optimisation of the (FFF) parameters and conditions is important to ensure maximum silica (%) recovery rates are obtained. As a result cross flow setting, injection time and field programme were (FFF) parameters that were investigated for the coffee creamer sample. In addition different buffers and carriers as well as enzymatic digestion were investigated to achieve separation of the silica nanoparticles from the complex matrix. The use of (Tris-HCl) as a buffer as well as a carrier was examined to see whether silica nanoparticles could be extracted from the coffee creamer matrix. In addition the results obtained from the (TEM) imaging was used to confirm whether the extraction of silica nanoparticles from the matrix was possible. Further work on characterisation of silica standards was performed to assess its behaviour and suitability for use as spikes for (IDMS) in the presence crude coffee creamer. Results for both Klebosol and NanoChop silica nanoparticles would reveal whether they have potential to become spikes for (IDMS). Furthermore the silica (%) recovery rates were determined to assess the extraction efficiency and loss of material due to the interaction with the membrane and or tubing.

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3. Abbreviations

BSI (British Standards Institution)

CEN (European Committee for Standardisation)

EFSA (European Food Standard Agency)

EUJRC (European Union Joint Research Centre)

ISO (International Organisation for Standardisation)

IUPAC (International Union of Pure and Applied Chemistry)

LGC (London Government Chemist)

NIST (National Institute of Science and Technology)

REACH (Registration, Evaluation, Authorisation and Restriction of Chemical substances)

Asymmetric Field – Flow Fractionation (AF^4)

FFF (Field – Flow Fractionation)

FIFFF-MALS (Flow Field Flow Fractionation coupled to Multi – Angle Light Scattering)

FFF-ICP-MS (Field – Flow Fractionation coupled Inductively Coupled Plasma)

ICP-MS (Inductively Coupled Plasma)

IDMS (Isotopic Dilution Mass Spectrometry)

MALS (Multi – Angle Light Scattering)

SEM (Scanning Electron Microscope)

TEM (Transmission Electron Microscope)

UV/Vis (Ultraviolet/ Visible light detector)

(Au^{197}) Gold

(Ge^{72}) Germanium

(Li^{7}) Lithium

(Rh^{103}) Rhodium

(Si^{28}) (Si^{29}) Silica Isotope

(Ti^{205}) Titanium

(Y^{89}) Yttrium

(NaCl) Sodium Chloride

(SiO_2) Silicon Dioxide

(TiO_2) Titanium Dioxide

(CTAB) Cetytrimethylammonium bromide (an ionic detergent)

(COOH) Carboxylic acid

(EDTA) Ethylenediaminetetraacetic acid

(HCl) Hydrochloric acid

(HF) Hydrofluoric acid

(PBS) Phosphate Buffered Saline

(SDS) Sodium Dodecyl Sulfate

(Zolt 1) Strober SiO_2 in ETOH (Ethanol) suspension silica nanoparticle

(Zolt 3) $SiOAR_4$ silica nanoparticle

(HSA) Human Serum Albumin

(I.E.P) Iso-Electric Point

(QC) Quality Control

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5. Introduction

The incorporation of manufactured nanomaterials into food products has grown exponentially due to its many advantages. A number of scientific articles and review have highlighted the applications of nanomaterials in the food industry, in particular *Ivask* provides a brief overview of the current nanotechnology in food consumer products.[1, 2, 3] However there have been safety concerns raised regarding the use of nanomaterials in food products, which was the possibility that nanoparticles could exert toxicity.[1, 4] As a result the need to develop standardised traceable methodology along with certified reference materials for the characterisation of nanoparticles in complex matrices has been much needed recently to provide quality assurance for the experimental work.[4, 5]

According to the ISO/TS 27687 definition for the term nano-objects, they are nanomaterials with at least one dimension within the nano-sized range (<100nm). Furthermore they specified the meaning of nano-object into the following. They are nanoparticles which have three, nanofibres which have two and nanoplates which have one dimension that are less than the 100nm size range.[6] The nanoscale size ranges between a lower limit and an upper limit of approximately; which was set at 1nm to 100nm.[4, 7] In addition the weight based cut off meaning the percentage (%) particle size distribution that contains the nano-sized particles in the collected fraction. Including agglomerated and aggregated nanoparticles the weight based cut off is set at 10%. However the problem is that some regulatory bodies define nano-objects differently. For instance a proposed definition of nanomaterial drafted by the European Commission Joint Research Centre (JRC) can also be obtained. This definition can be used for adopting and implementing regulations regarding nanotechnologies in consumer products. The (EU) definition for nanomaterials was that they consists one or many dimensions of less than 100nm. In addition nanoparticles unbound aggregated or agglomerated where 50% of its size distribution consists of dimension between the ranges of 1-100nm. The variation between the ISO and (EU) definitions was at their weight based cut off.[7, 8] As a result there are some slight differences in the nanoparticle definitions, which pose a problem for regulators and industries, but as mentioned by *Ivask* a revise of the (EU) definition will soon take place in 2014.[8, 9,]

Furthermore studies on certain nanoparticles due to their unique properties compared with bulk materials there has been concerns of possible toxicity when these nano-sized objects are consumed. Importantly the consequence of the long term exposure of nanoparticles on human and animal health is still not fully understood.[10, 11]

The characterisation of nanoparticles in complex food matrices has become a major challenge. As explained by *Barceló and Farré* currently very few work on the characterisations of nanomaterials have been applied in the presence of complex food matrices, particularly in cases whereby nanoparticles that exist in very low concentrations. The issue with complex food matrices are that they contain a wide range of different natural and engineered nano-size structures which are integrated into the matrices this increases the difficulty of separating and detecting the nanoparticle. As food matrices can also contain a variety of functional macromolecules for example lipids, proteases, carbohydrates and specifically in coffee creamer milk micelles (20-300nm) and fat micelles (1-2µm) which could obscures and interferes with the analytical signal coming from the nanoparticles themselves.[1, 11] Furthermore the appropriate methods in separating the nanoparticle from the food matrices either via physical or chemical means must be selected with care. This was because reducing or changing nanoparticles surrounding such as altering the matrices could potentially affect nanoparticles properties and behaviour.[12] What's more during the analysis of the nanomaterial, interactions could potentially occur between the nanoparticles and the food matrices.[1] This was because nanomaterials have unusual properties compared with larger bulk materials such as their larger surface area to volume ratio that causes them to have a tendency to agglomerate. This certainly adds to the complexity of analysing nanoparticles in food.[13, 14] In order to tackle the problem of possible aggregation and problem of matrices, a combination of characterisation methods will be required in order to successfully characterise the nanoparticles.[1, 13] *Tiede* and *Kammer* highlights some of the issues in the characterisation of nanoparticles in complex media these include:[15, 16]

- reduced extraction and recovery rates of nanoparticles due to sticking
- presence of artifacts caused by sample preparation
- distinction engineered and natural nanoparticles
- lack of certified reference materials
- possible alteration of nanoparticle when matrix environment changes

The European Union and international legislations for example (REACH) (1907/2006) and Regulation 178/2002 put in place as a precautionary measure are considered to be adequate in regulating the use of nanotechnology in the food industry thou new changes regarding nano-safety are continually being implemented.[10, 17] The development for a standardised methodology in the characterisation of nanoparticles in complex matrices are vital for international regulatory bodies to implement and support legislations regarding the use of nanomaterials particularly in consumer products such as labelling requirements to specify products containing nano-object as well as for providing quality control for products placed on the market. For instance the European Food Standard Agency (ESFA) ensures

that the food products safety assessments have been met prior to it being marketed.[17, 18] However the process of developing standardised methodology is a time-consuming process, many of the development work carried out by standardisation bodies such as (ISO) and (CEN) has focused on the characterisation and safety assessment of the nanomaterial instead of quantification of nanoparticle in complex matrices.[17, 19] Examples of the international regulatory bodies and legislations currently established regarding the use of nanotechnology in industries were given elsewhere.[20] Furthermore as mentioned by *Stamm* under the guidance documents provided by the (IUPAC) and Commission Decision 2002/657/EC7, methods for the characterisation of nanoparticle should in principle carry out method performance parameters which includes recovery rates, reproducibility and repeatability of each sample. This was because of possible measurement uncertainties that have been reported whilst performing characterisation studies on nanoparticles. High measurement uncertainties could be obtained during the first few sample runs. This could be because of several factors:[19]

- incorrect sample preparation
- incorrect calibration of instrument
- lack of standardised protocols available
- could be down to instrument interferences

This could ultimately influence the reproducibility of the results. However as summarised by *Dekker*, the work on the characterisation of nanoparticles in food matrices is of great importance because it will give vital information for future studies which looks into potential nanoparticle toxicity which at the moment is currently lacking.[21, 22]

In the chacterisation of nanomaterial there are four crucial analytical steps that takes place:[15]

- firstly detecting the presence of the nanoparticle,
- secondly identifying the specific type of nanoparticle present,
- thirdly separation of the nanoparticle from its matrices,
- finally quantifying the fraction of nanoparticle present in the sample.

However as noted by *Weigel*, characterisation of the nanoparticles becomes a problem when nanoparticles are present in complex matrices. This means that nanoparticles must be separated from the matrix first, before the characterisation of nanoparticles can begin.[16, 23]

Indeed previous research on the characterisation of nanomaterials in complex matrices had been performed using a variety of different techniques some recent examples

were highlighted elsewhere.[20] *Tiede* mentions due to the lack of methodology available for characterisation on nanoparticle in complex matrices this has hindered a lot of the scientific progression. Nevertheless they provided a review on the progress of works carried out on the characterisation of engineered nanoparticle in complex media.[16] Moreover *Kammer* highlighted some of the progress made on the methods regarding nanoparticles in complex matrices, such as sample preparation and techniques for the detection of the nanoparticle.[15] Furthermore the use of (FFF-ICP-MS) for the analysis of nanoparticles elemental and size characterisation has now become a promising and versatile choice of instrumentation. As well as the ability to quantify the amount of silica nanoparticles present in the sample.[18] In addition work by *Weigel and Peters* concluded that (FFF-ICP-MS) is a very capable instrumentation for the analysis of inorganic nanoparticles in complex matrices; their work was focused also on silica nanoparticles with coffee creamer. Although they noted that sample preparation can be an issue and highlighted that nanoparticle interaction with the matrices should be investigated further. To ensure that nanoparticles are separated from the matrices they selected the use of enzymatic digestion compared with the choice of using harsher chemical digestion. They also observed silica nanoparticles in coffee creamer matrices agglomeration under the view of transmission electron microscope (TEM). In addition they advised the use of well established, stable standards and reference materials to be used as quality control throughout the experiment.[23] The popularity in (FFF-ICP-MS) instrumentation for characterisation work is because of (FFF) ability to perform high resolution separation down to 2-200nm range; fractionating the nanomaterials into different nanoparticles sizes then by using (MALS) nanoparticles can then be characterised into their particle size distribution. Finally the introduction of the separated sized fraction into (ICP-MS), an instrument that provides high specificity and sensitivity down to part per billion levels for the elemental analysis of the nanoparticles which are essential for later future work.[24], [25]

6. Aim

For the experimental work the objective was to compare several methods developed by (LGC) earlier, for the characterisation and quantification of silica nanoparticles corresponding to the ISO and (EU) definition for nanoparticles in the presence of complex matrices. In the experiment coffee creamer samples was investigated. Whether silica nanoparticles are present in the coffee matrix if so are they nano in size range. The method was based on previous protocol set out by *Goenaga-Infante, H.* and co-workers using asymmetric (FFF) coupled with (ICP-MS) which has been adapted for this experiment. This involves employing the use of (MALS) detector connected to the FFF-ICP-MS instead of just

UV-Vis detector on its own to enable the size measurement of the silica nanoparticles.[5] In addition the use of (TEM) microscopy work was to compare and contrast the changes for the samples in (Tris-HCl) buffer and for those in water which was eluted from the (FFF-ICP-MS) instrumentation.

The initial part of the experiment was to carry out optimisation of the experimental conditions for the silica samples eluting from the (FFF-ICP-MS) instrument which were based on methods developed by (LGC) earlier. The optimisation of experimental conditions was performed by selecting different (FFF) parameters i.e. crossflow, injection time, and field programme to ensure that maximum (%) recovery rates can be obtained. In addition optimisations of (FFF) solvents were also performed selecting different carrier fluid for (FFF) as well as choice of buffers for sample to use for the instrument. Once the optimal conditions were established the focus was on the quantification of the silica nanoparticles in the coffee creamer matrix using post (FFF) calibration approach based on *Nischwitz* protocol.[26] The assessment of the silica behaviour and conditions eluting from the (FFF) was performed by determining the (%) recovery rate. Furthermore coffee creamer were spiked with two different silica nanoparticles and behaviour of the spikes as well as with the native silica was investigated, in order to choose a better spike for future (IDMS) experimental works.

7. Experimental

7.1 Equipment

7.11 (FFF-ICP-MS)

A schematic representation of the set up can be seen (below) in *fig 7A.* The experiment was conducted using the AT model AF2000 Multiflow (FFF) [1] instrument manufactured by Postnova Analytic. Split up into various compartments; PN4020 serial number 090710-050, temperature set up; PN7140 solvent organiser; PN 7520 solvent degasser; PN 1130 focus isocratic pump; PN1130 tip isocratic pump; AF2000 Flow (FFF), and the injection of samples were introduced into a 21.8µl volume injection loop.[24] Detection achieved by MALS PN3621 as well as (UV/ Vis) ACCELA PDA detector manufactured by Thermo. These are connected onto a DELL Optiplex manufactured PC. Two instruments (ICP-MS) were used during the experiment. One of the instruments used was (ICP-MS) [5] Agilent series 8800 triple quad manufactured by Agilent technologies, serial number JP12480146. The main bulk of the experimental work was performed by (ICP-MS) series

7700 [3] connected with a ASX5000 autosampler also manufactured by Agilent technologies, this was connected to a HP Compaq PC, that uses a HP laserjet printer.

7.12 Carrier filtration

The (FFF) carriers used during the experiments were all filtered through Millipore filter funnel apparatus, ID number ID40/35. This was connected onto Laboport electrical pump. A pump filter with 1µm pore size, ID number 0.0966FIPA16734 was used. Carrier solutions were filtered through filtration paper manufactured by Postnova with a membrane pore size 0.1µm 47mm diameter.

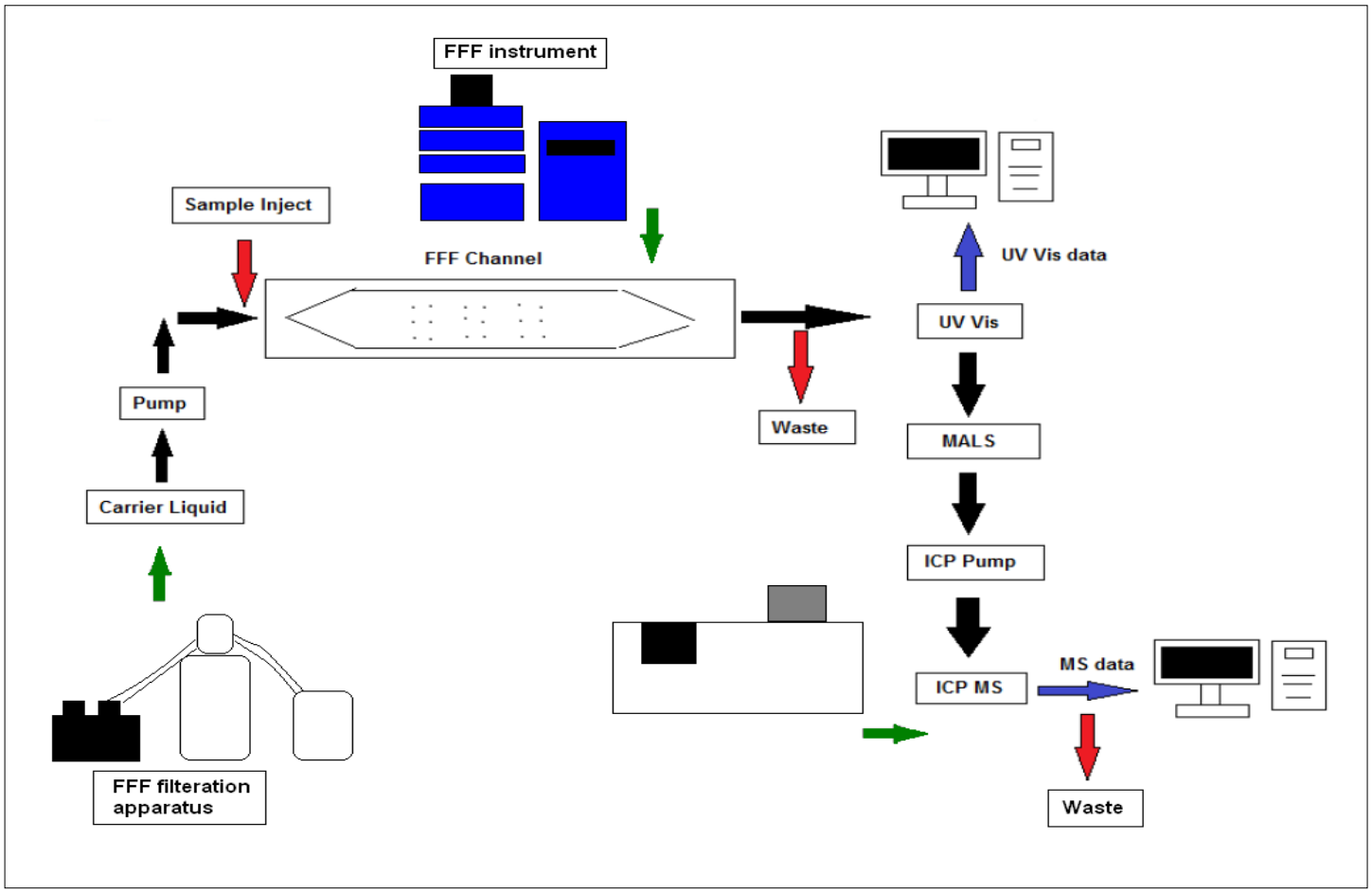
7.13 Apparatus for (FFF) channel

An asymmetric field flow fractionation (AF^4) channel [3] was used for the experiment; model number CHA612, serial number 020412-012 WEEE. The dimension of the channel consists of membrane pore size of 10KDa and a thickness of 350µm. To open up the membrane channel Postnova Analytics equipment such as the Prozzon Micro click, part number Z-AF4-200-12 was used.

7.14 Other

The solution pH was checked with Fisherbrand hydrus pH meter. Samples were weighed using Denver TC-2102 balance, GC number: 010105. Daily calibration checks are made using National Physics Laboratory UKAS calibration weights, serial number: 08181. Solutions were pipetted using Gilson brand pipettes; P9 for 1000µl and P18 for 1-5ml. Samples were shaken using Microthermo. Manual injection of samples into (FFF) performed using Postnova Analytics 100µl injection syringe, part number Z-HAM-81075.

Figure 7A: Schematic representation of the FFF coupled ICP-MS instrumentation connected online to UV Vis and MALS detectors adapted from ref [18]



7.2 Samples and Reagents

7.21 Certified reference materials

Certified reference materials are important to ensure precise measurement to verify and evaluate the performance of the instrument, this act as the quality control run before the start of the sample runs. The (Au) standards used for the experiments are purchased from National Institute of Science and Technology (NIST). Three different sizes of (Au) reference materials were used they were RM8011 (Au) nanoparticles which has a nominal diameter of 10nm; RM8012 which has a nominal diameter of 30nm; RM8013 which has a nominal diameter of 60nm. The suspensions were prepared daily using 1 in 50 dilutions of 10, 30 and 60nm gold NP mix Elga water.

7.22 Tuning solutions for (ICP-MS)

Tuning of the ICP-MS sensitivity was carried out daily against the (ROMIL) tune solutions: 1ppb (standard mode) and 10ppb (hydrogen mode).

7.23 Post-FFF calibrants

Silicon and Titanium (ROMIL) mix at 250, 500, 1000 and 5000ng/g were prepared in 5% nitric acid (Sigma-Aldrich) Containing 10ppb of (Ge), (Y) and (Rh).

7.24 (FFF) Carriers

Several carriers were investigated in the experimental work these included water, Tris-HCl and (SDS). The ultra-pure water (Purelab flex Veoliathis was set at 18.2Ωcm^{-1} 25°C). Tris-HCl (was prepared from TRIZMA base (Sigma-Aldrich) at 50mM concentration in ultrapure water, with pH adjusted to 7.5 with HCl (Sigma-Aldrich, product code T4661, Lot number MFCD00004679) Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, product code L3771, Lot number MKBP2430V) at 50mM was prepared in ultrapure water. As mentioned previously all (FFF) carrier solutions are filtered through 0.1µm membrane.

7.25 Coffee Creamer

Coffee creamer brought from general convenience store. To obtain filtered sample the solution was passed through different filtration sized syringes. The following sterile disposable filtration syringes were used they include 5µm Minisort satorius stadium (cellulose nitrate base), Lot number: 1759420416103; 0.45µm Whiteman, code number CA W/QMF; 0.2µm Whiteman code number CA W/QMF and 0.1µm Millex Durapore PVDF membrane, Lot number: RIPA6919.

7.26 Silica nanoparticles

Silica nanoparticles used in the experiment included Nano-Chop 01 Colloid Silica ID vial number 0239 (RM1). (Zolt 1) Strober SiO_2 in ETOH aqueous solution, Lot number: 2 and

(zolt 3) ($SiOAR_4$) silica standards containing arginine. In addition NanoChop 02, Lot number 0121 and Klebosol 11, Lot number were also examined. Silica samples were diluted with either a 50 or 100 times, according to the amount of silica required by the (FFF) to enable detectability.

7.27 Enzymatic reagents

Two different types of enzymes were used in the experiment. Proteases from *Streptomyces* P5147-1G, Lot number: 110M1644V and Lipase from *Candida Rugosa* L1754-2SG, Lot number: 1406749 55108109 which were manufactured by Sigma-Aldrich. The sample coffee matrix was digested into a 2:1 ratio of protease and lipase enzyme mixed with the selected buffer solution.

7.3 Procedures

7.31 Enzymatic digestion of coffee creamer

An aliquot of 0.5g coffee creamer was placed in a 20ml Falcon tube along with 20ml of Elga water (or 20ml of the selected buffer. Three types of buffers were used in this study: (Tris-HCl) pH 9, (Tris-HCl) pH 7.5 and ammonium acetate pH 7.5. Coffee creamer suspension was vortex for 1 min prior to enzymatic digestion ('crude') or filtered through a decreasing pore sizes syringe filters ('filtered', see section 7.25). Enzyme mix containing 2:1 ratio of protease: lipase was prepared in the selected buffer. Specifically 0.05g protease and 0.025g lipase was weighted in 15ml Falcon tube and suspended in 2.5ml of the selected buffer. Next, the enzyme mix (250µl) was added to the coffee creamer (250µl) was and incubated for 4- 24hrs at 37°C, with shaking.

7.32 Spiking of the coffee creamer

An aliquot of 1g coffee creamer sample was added to 50ml plastic tube. Next 10ml of the mixture was transferred into two separate 15ml plastic tube. In of the one tube 100µl of Strober SiO_2 standards was added. The other tube was spiked with 100µl of ($SiOAR_4$) standards.

7.33 Spiking of the samples

An aliquot of 1g coffee creamer sample was added to 50ml plastic tube. Next 10ml of the mixture was transferred into two separate 15ml plastic tube. In of the one tube 100µl of Strober SiO_2 standards was spiked. The other tube was spike with 100µl of ($SiOAR_4$) standards. After that an aliquot of 2:1 ratio; 0.05g of proteases and 0.025g lipases enzymes was weighted into 15ml plastic tube and 5ml of (Tris-HCl) at pH 7.5 buffer was added. This enzyme mixture was transferred into four separate ependorff tubes. In of the one tube 500µl of the enzyme mix along with 500µl (Tris-HCl) pH 7.5 was added to create a blank. Next tube 500µl of the enzyme mix along with Strober SiO_2 standards was added to create enzyme digested Strober silica sample. In another 500µl of the enzyme mix along with 500µl of ($SiOAR_4$) silica standards was added to create enzyme digested ($SiOAR_4$) sample. Finally 500µl of the enzyme mix along with 500µl of crude coffee creamer was added to create enzyme digested crude coffee creamer sample. In addition a 1/100 dilution factor for both Strober (SiO_2) and ($SiOAR_4$) silica standards were made separately. 1ml of the (Tris-HCl) was added into ependorff tube which was spiked with 100µl of the selected silica standards.

7.34 Silica standards

Added 10ml of the Elga water into a 15ml plastic tube this was spiked with 0.01g of either the Strober silica standards or ($SiOAR_4$) silica standards to create the silica standard samples. In addition an aliquot of 0.5g of coffee creamer with 20ml of water was made. This mixture was transferred into 10ml plastic tube that was spiked with the ($SiOAR_4$) to create silica standards in presence of coffee creamer.

In addition for the NanoChop 02 and Klebosol silica standards both 1/50 and 1/100 dilution factors were performed. Into an ependorff tube, 10µl of the silica standards for 1/100 dilution and 20µl for 1/50 dilution were pipetted with 1ml of Elga eluent. Klebosol silica standards in (Tris-HCl) at pH7.5 buffer were also prepared 20µl of the Klebosol standard was pipetted with 1ml of 50mM (Tris-HCl) at pH 7.5.

7.35 Total Si28 and Si29 analysis.

Weighted 0.1g of the coffee creamer sample, 0.01g silica standards, 0.5g silica intermediate at 10 µg/ml, along with 1g blank, that were digested into a mixture of 25µl hydrofluoric acid (HF), 4ml (10%) nitric acid, 4ml (10%) hydrogen peroxide and 1ml water

eluent using a microwave system in vessels. Finally 40 ml of water added into the digested mixture for ICP-MS total Si28 and Si29 analysis.

Total silica nanoparticles counts were performed by adapting from *Nischwitz, V and co-workers* protocol, for the determination of the concentration of silica in µg/g as well as for the percentage extraction efficiencies of the methodology.[25, 27] Microwave digested samples including digested QCs were introduced directly into (ICP-MS) using autosampler. They were run along with external internal standards that contained (Rh), (Ge) and (Y) with 7% nitric acid silica isotopes 28, 29 and 30 were monitored in hydrogen mode. In addition calibration points at 0, 250, 500, 100, 5000ng/g were introduced between the start and end of the run to monitor drifts, but also acted as a validation to the methodology.

7.36 *TEM Analysis*

Transmission electron microscopy (TEM) was used to compare crude coffee creamer samples in the presence of water to that of Tris-HCl pH7.5 buffer. The eluting (FFF) fraction was collected at specific time point for both silica and titanium nanoparticles into an ependorff tube. Two different types of (TEM) sample were prepared, firstly (TEM) sample that were collected directly from (FFF) and secondly (TEM) sample that were concentrated using a spin filtered tube containing a 10Kda membrane. (TEM) grids were purchased from Agar scientific, code: S160-4H; 400 mesh copper film grid. The individual (TEM) grid was laid on top of a paraffin film. For the non concentrated (TEM) sample 15µl of the (FFF) fraction was dropped onto the grid which was left to dry. For the concentrated sample 500µl of the (FFF) fraction was pipetted into a spin filtered tube. This was spun at 13,400rpm at room temperature for 15mins. Finally 15µl was pipetted onto the grid which was left to dry ready for viewing.

7.37 Analysis of coffee creamer sample employing (FFF-ICP-MS)

The method used for the analysis of the coffee creamer sample was adapted from previous work performed by *Goenaga-Infante, H.* and co-workers, their work on experiment the characterisation of (TiO_2) in sunscreen.[27] The separation of the nanoparticles into their sized fractions was carried out by using Postnova Analytics AF2000 MT model asymmetric field flow fractionation (AF^4). The (FFF) membrane channel consists of regenerated cellulose that has a pore size molecular weight of 10KDa along with a spacer thickness of 350µm.

The (FFF) carriers used were all filtered through a 0.1µm pore size membrane using the filtration apparatus. Water carrier was used quite extensively, but other carriers were also used to study the effects it has on coffee creamer samples. Samples were mixed thoroughly before introduction into (FFF). Sample injections were performed by manual injection using Postnova Analytics 100µl injection syringe into the 20µl injection loop. For most part of the experiment the (FFF) parameters were set at the following settings. Injection time 4 minutes, 0.2 ml/min tip flow, 1.3 ml/min focus flow and detector flow rate kept at 0.5ml/min. However the cross-flow rate applied on the (FFF) and the field programme changed during some parts of the experiment. The (FFF) fractograms produced gave the elution profile of the injected sample, characterising the nanoparticles based on their size distribution. Addition information can also be revealed such as whether nanoparticles were aggregated, from the presence of a large peak late in the fractograms or whether loss of sample occurred, presence of a large void peak. Moreover the (FFF) software can evaluate the size of the nanoparticles, after the nanoparticles are separated into their size fraction.[18, 24, 28] The performance of the (FFF) was carried out by using NIST (Au) certified reference materials using water as (FFF) carrier, employing Agilent ICP-MS 7700 to monitor internal standards (Rh), (Y), and (Ge), with (Au) in standard mode as a daily quality assurance check.[29] For sample runs internal standards were also used which contained internal standards (Rh), (Y) and (Ge) that were mixed with the eluent. In addition the elements (Si), (Ti) and (Au) isotopes were monitored in hydrogen mode to monitor any drifts in the instrumental run.[27]

7.4 Quantitative Analysis

7.41 Quantification and recovery of silica nanoparticles in samples

The protocol set up by *Nischwitz, V and co-workers* was adapted for the quantification of Si nanoparticles in the samples eluting from the (FFF) instrument. Their work involved the analysis of metal contents in serum and cerebrospinal fluid.[26] They performed multi element-calibration method whereby they measured the flow rate of (5%) nitric acid that contained internal standards (Rh) and (Y) and (Ge) at 10µg/L to normalise isotope peak intensities. In addition other metal isotopes Manganese (Mn), Copper (Cu), Iron (Fe) and Zinc (Zn) at 0, 0.5, 1, 5 and 10µg/L were added in, which they were monitoring in their studies. This enabled the mass flow of the metal contents going through the (ICP-MS) to be calculated and correlated according to the peak intensity in order to quantify the metal contents present in their sample. However for this experiment the metal isotopes were replaced by silicon and titanium standards at 0, 250, 500, 1000, 5000ng/g in 5% nitric acid. To perform the quantification each calibration point were injected directly into the (ICP-MS)

to create a mass flow chromatogram.[26, 27] The amount of nanoparticles loss during the sample introduction into (FFF-ICP-MS) was determined by calculating the recovery rate. This can be done by direct injection of sample into the (ICP-MS) and comparing with the (FFF) injected sample.[30]

7.42 Total silica nanoparticles in coffee creamer

Total silica nanoparticles counts were performed by adapting from *Nischwitz, V and co-workers* protocol, for the determination of the concentration of silica in µg/g as well as for the percentage extraction efficiencies of the methodology.[26, 27] Microwave digested samples including digested QCs were introduced directly into (ICP-MS) using autosampler. They were run along with external internal standards that contained (Rh), (Ge) and (Y) with 7% nitric acid, silica isotopes 28, 29 and 30 were monitored in hydrogen mode. In addition calibration points at 0, 250, 500, 100, 5000ng/g were introduced between the start and end of the run to monitor drifts and to validate the methodology.

8. Results and Discussion

8.1 Quality Control

8.11 (Au) Certified Reference Materials

Quality control of results was performed by assessing the (FFF-ICP-MS) performance by using (Au) certified reference material. In the appendix part A section, *fig 10.1A and 10.1B* shows the (FFF-ICP-MS) fractograms for the reference material (Au) nanoparticles during the course of the experimental work. The results obtained were very consistent; one point to highlight was that (MALS) fractogram and the (ICP-MS) signal were slightly different each day. This could be as mentioned by *Goenaga-Infante, H.* and co-workers, the slight shift in the elution profile and (ICP-MS) signal could be due to the age and reduced stability of the gold nanoparticle, as (Au) nanoparticles are not very stable itself.[27] With (FFF) the nominal 60nm (Au) could not be detected due to light scattering interferences from the larger particles, compared to 10nm and 30nm which were both detected at 15mins and 20mins respectively as shown in *fig 10.1A*. However with (ICP-MS) higher sensitivity and removal of light scattering interference allows 60nm to be detected at 20mins shown in *fig 10.1B*. As a result the daily assessment of the instrument performance was carried out by running (Au) certified reference materials. From the literature search the (Au) nanoparticles are coated in a citrate layer to minimise particle interaction. The reported

average size of the (Au) certified reference materials were 13.5+-0.1nm, 28.6+-0.9nm and 56.6+-1.4nm, using dynamic light scattering technique suspended in aqueous solution.[31] Using the (FFF) software the size of both the 30nm and 60nm gold standards were verified to be approximately 15nm in radius and 30nm in radius respectively. The advantage of this method is that it is a simple checking procedure in addition this procedure gave high (%) recovery rates which was reported to be approximately 85-96%.[27]

8.2 Method Optimisation - based on earlier protocols developed by (LGC)

8.21 (FFF) parameters

As mentioned by *Messaud*, due to the principles of (FFF) theory, there will be differences between the experimental results obtained compared with the expected results, based on the theoretical assumptions of how nanoparticles would behave. This could be because of:

- zone broadening
- nanoparticle - (FFF) membrane/ tubing interaction
- non-uniform field strength
- sample overloading
- nanoparticle - nanoparticle interaction
- solvent effects

The above effects can be minimised, apart from zone broadening by controlling different (FFF) parameters. Such as flow rates, sample and solvent concentration, temperature. The use of light scattering detectors can also lessen the effects as it offers independent measurements of the size and molecular weight of nanoparticles.[32]

Therefore the choice of (FFF) parameters will have direct influence on the separation of the nanoparticles from its matrices as well as impacting the recovery rates of the nanoparticles. This was demonstrated by *Whitley* in her experimental work on method development for the characterisation of silver nanoparticles in environmental soil samples using (AF^4), whereby crossflow injection time and fractogram runtime settings were investigated.[33] In addition as mentioned by *Bolea,* their experimental work investigated the effects of various (FFF) parameters and conditions that effected the stabilisation of Silver (Ag) nanoparticle, they noted that the injection and focusing steps of the (AF^4) could potentially change the nanoparticles surroundings and behaviour, leading to potential aggregation of nanoparticles. As a result efforts must be made to ensure conditions set by

(AF^4) do not promote aggregation of nanoparticles. The overall picture was that optimisation of the (FFF) parameters are essential to ensure that the conditions applied favours the separation of silica nanoparticles from its complex matrix as well as for achieving good recovery rates.[34]

As shown (below) in *fig 8A* these were some of the main (FFF) parameters that were looked at for method optimisation based on earlier work developed by (LGC). This would allow optimal (FFF) parameters for the coffee creamer sample to be determined. The focus was on optimising the injection time, cross-flow and the field programme on (FFF) as these settings were previously investigated by (LGC).[5]

For the experimental work the fractogram runtime time was optimised, the runtime of 35mins was considered to be suited to the silica nanoparticles. Runtime lower than 25mins was believed to be too short, as the fractogram results obtained clearly shows that the samples was not completely eluted. However for new samples introduced the first time into the instrument, a runtime of 55mins was selected to gain a complete elution profile of the sample in which a decision was made later as to whether the runtime could be adjusted lower to give faster analysis time. As summarised by *Qureshi and Kok* method optimisation will involve finding a compromise between the speed of separation as well as the detectability of the samples whilst they were working on characterisation of proteins using (AF^4).[35]

The injection time was optimised, injection time 4mins was considered to be appropriate for the nanoparticles shown in the appendix section part A *fig 10.2A,* good separation of the nanoparticles was achieved as shown from the sample peak between 20-30mins, as large particles from the matrix were separated from the nanoparticles after 25mins. Furthermore the void and sample peak were both well resolved, a small void peak can be seen at 5mins. Injection time of less than 4mins produced undesirable fractogram because of a larger void peak present as well as peak appearing before the void peak, indicating potential loss of nanoparticles. In addition there was no indication that large particles eluted after 20mins, meaning that separation of the nanoparticles from the matrix was poor or it could be that no separation occurred as shown in *fig 10.2C*.[23] This could be explained by *Qureshi and Kok,* typical samples that have low diffusion coefficient will require longer injection time, which was necessary to ensure that solvents had enough time to flush and rinse through the (FFF) tubing to prevent any sticking, thus obtaining smaller void peaks. In addition more time should be given for the (FFF) relaxation process so that samples can be focused at the injection port.[35, 36]

Figure 8A: Summary of the selection of (FFF) parameters investigated as part of method optimisation based on earlier work by (LGC) adapted from ref [30]

FFF system		Postnova Asymmetrical AF2000 MT model
Membrane		10KDa regenerated cellulose
Channel flow (ml/min)		0.5
Injection time (min)	trial 1	4
	trial 2	3
	trial 3	1
Crossflow (ml/min)	trial 1	1
	trial 2	1.5
	trial 3	2
	trial 4	2.5
	trial 5	3
Fractogram runtime (min)	trial 1	25
	trial 2	35
	trial 3	60
Field programme (elution step)	trial 1	Constant
	trial 2	Linear decay (20mins)
	trial 3	Parabolic
UV-Vis detector		Thermo
Wavelength (nm)		280nm and 520nm
MALS		Postnova
Angles (˚)		21 different angles present
ICP-MS		Agilent 7700 series
Nebuliser and Spray Chamber		Micromist and Scott spray chamber
Masses monitored		Au^{197}, Si^{28}, Si^{29}, Ti^{46}, Ti^{49}, Ge^{72}, Y^{89}, Rh^{103},
Flow rate (ml/min)		1
Nebuliser rate		0.5 rps (revolution per seconds)

Another crucial (FFF) parameter that was optimised was the cross-flow. The fractogram obtained revealed that high cross-flow setting at 2.5ml/ min provided better separation between the silica nanoparticle and the food matrices shown in *fig 10.2E*, showing silica nanoparticles between 20-30mins and the larger matrix materials eluting at 25-30mins. This was compared with low cross-flow setting shown in *fig 10.2G* that had a silica peak between 20-25mins, but larger particles from matrices cannot be observed after 25mins. This could suggest that the nanoparticles and the food matrices did not separate well enough and that the peaks could not be clearly distinguished from one another.

Furthermore the crossflow setting directly affects the resolution of the fractograms. This could be explained by (FFF) theory as shown (below) in *fig 8B* the equation shows how the retention time relates to the diameter size of the nanoparticles providing that a linear

relationship is established between the experimental retention ratio of $R=t_o/t_r$ resulting in the equation $R=6\lambda$, which was covered by *Wahlund and Nilsson* on pages 6-8.[37] Importantly it shows how different (FFF) parameters i.e. crossflow can have an effect on the retention time. This theory can be mathematically interpreted to give a fractogram output which yields the information on the nanoparticle size distribution.[34, 35] However the theory does assume that nanoparticles are all similar in shapes and sizes.[38] Furthermore as mentioned by *Bolea*, cross-flow plays a role in the quality of the separation. They noted that a high F_c value (crossflow rate) referring back to *fig 8B*, gave better separation. Thus resulting in less band broadening that gave narrower fractogram peak; thus resulting in higher peak resolution. As demonstrated by *Bolea*, with their work, they shown that with high cross flow at 0.325ml min^{-1}, they obtained a R value of 1.12 compared to lower cross flow at 0.2ml min^{-1}, R=0.60, ideally R should be 1.5 to be considered as a good resolution, hence better separation obtained. In addition they noted that injection time did not have direct influence on the peak width.[34] Moreover as noted by *Messaud*, they mentioned another fact in which (FFF) relaxation and focusing parameters could also influence resolution. On a side note they mention how elevating the temperature of Flow (FFF) can also decrease band broadening, enhancing separation and reducing analysis time.[32] On the contrary *Qureshi and Kok* highlighted the fact that a higher cross flow setting does have its limitation because of the instrument limitations. Such as possible leakage from channel, as smaller nanoparticle could potentially be force through permeable membrane due to the stronger crossflow force. Or samples could become attached onto the (FFF) membrane channel resulting in sample losses as well as prolonging analysis time.[35] This was demonstrated by *Baalousha, their* work on natural colloid particles using (FIFFF-MALS). They illustrated that with low cross-flow setting, the fractogram obtained had a much poorer separation as well as lower retention time obtained. But higher crossflow setting resulted in selective loss of some samples due to the stronger force of the cross flow.[39] Another problem mentioned by *Qureshi and Kok* was the possibility of sample overloading at higher cross-flow setting particularly with concentrated samples. Moreover concentrated samples tend to produce wider and broader peaks due to higher tendency for electrostatic interaction between the nanoparticles and sample materials. They highlighted the fact that the focusing step of the (AF^4) plays an important role in limiting the problem of sample overload. Their solution to the problem was to perform dilution before injecting the samples in (FFF) and to optimise the (FFF) focusing parameters prior to analysis. Making sure that samples do not become too diluted as this would affect sample detectability because of limit of detection. In addition the focusing step should not be too short as resolution will be affected nor should it be too long as smaller molecular weight samples could be lost within the permeable membrane channel.[35]

Figure 8B: FFF theory showing the relationship of the σ_t retention time to the nanoparticles molecular size, where *w* height of the channel and the thickness of the spacer, u_c cross-flow velocity and (F_C/F_{OUT}) flow ratio; F_c cross flow rate and F_{out} flow rate out of channel, formula adapted from ref [35]

$$\sigma_t = 0.82 \frac{w}{u_C} \cdot \left\{ \ln\left(1 + \frac{F_C}{F_{OUT}} \right) \right\}^{1/2}$$

As summarised by *Qureshi and Kok,* determing the optimal cross-flow setting can be difficult as it is also dependent on the type of sample i.e. matrices and nanoparticles and how they behave with the (FFF) membrane channel. They mentioned the fact that a shorter retention time was desirable, as analysis time would be shortened. As a result they suggested that the use of field flow programme. This means that crossflow force can either be increased or decreased gradually during the (FFF) sample run.[35] *Wahlund and Nilsson* mentions the advantages of using the field programme, in particular for situations where nanoparticles have a range of different sizes. In addition it is particular useful for obtaining a general elution profile of unknown sample composition or for initial sample runs.[37] There are three common field flow programme constant, linear and exponential. With the constant mode meaning the crossflow remains constant throughout. *Qureshi and Kok* mention the disadvantage with this mode. Where with high molecular weight samples, retention time could become too prolong, indication that the samples are residing within the membrane channel and increasing the overall analysis running time. However this mode is particularly useful in situation where new samples are being introduced and an overall elution profile is needed as shown in *fig 10.3A* and *fig 10.3B* in appendix part A. On the otherhand with linear decay mode meaning the crossflow stays constant for a while and then decreases gradually to zero. The advantage with this mode is that it would allow samples specifically larger particles to elute quicker giving a shorter retention time which was demonstrated in *fig 10.5A* compared with *fig 10.5A*. Finally with exponential mode where crossflow never reaches zero, is particularly useful for quick analysis of unknown sample this mode was not used extensively for the experimental work.[35, 37]

Overall injection time 4mins was considered optimal runtime for sample injecting into (FFF). In addition crossflow 3ml/min allowed separation of the nanoparticles from the sample matrices. Furthermore running samples with the linear decay setting allows larger particles in the sample to elute much quicker therefore reducing the analysis time.

8.22 Carriers, buffers and pH

A selection of different buffer at optimal pH value should be investigated as mentioned by *Nilsson*. This could also be extended to include salt concentration and surfactants being added into the carrier solvents. This was because depending on the selection of pH and buffer used for the carriers it could potentially affect the behaviour of nanoparticles. Such as the nanoparticle retention time due to the changes in the electrostatic properties of the nanoparticles because of the different carrier compositions at different pH.[40] Furthermore buffers as well as its pH can also play an influential role on the enzymatic activities. This was demonstrated with the work on lipase activity from *Candida rugosa* using a selection of different buffers at different pH that was highlighted by *Bellucci*.[41]

From the experimental results obtained, the use of water as buffer for the coffee creamer sample did not appear to have resolved as both the void peak at 5mins and sample peak at 10-25mins were very close to one another. This could be because the sample was not retained long enough to observe any separation or could be because samples were being lost in the void peak at 5-10mins as shown in *fig 10.3A*. Tgus was compared with (Tris-HCl) buffer at pH 7.5 and 9 shown in *fig 10.3C and 10.3E,* which had noticeable difference. As it appears to show an indication of separation between silica at 5-10mins and titania at 20-25mins. In addition a larger peak was observed at 20-30mins that appears to be the matrix eluting from the sample. Furthermore this was verified by the ICP-signal which shows a release of titania from the matrix at 20-30mins. This could be due to electrostatic influences exerted by (Tris-HCl) and ammonium bicarbonate buffers. This was demonstrated in the characterisation work on protein using (AF^4) performed by *Song,* they highlighted how the ionic strength of carriers affected the electrostatic properties of the protein molecules. They mentioned that as solvent ionic strength increased the electrostatic repulsion between the charged molecules decreases as a result the molecules migrates closer to the membrane wall therefore resulting in much longer retention time, this could explain the reason why there were differences in the retention times of the coffee creamer sample in the presence of different buffers.[42] Furthermore comparing the pH for (Tris-HCl) buffer at pH 9 and pH 7.5 as shown in *fig 10.3C and 10.3E,* there does not appear to be any noticeable differences in the MALS fractogram obtained, although void peak appears to be more

prominent for (Tris-HCl) at pH 7.5, however there was no clear indication that both silica and titania nanoparticles were separating from the matrix as both Si and Ti peaks on the ICP-MS were overlapping each other between 20-30mins. Looking closely at the ICP-MS signals obtained for both pH, as shown in *fig 10.3D and 10.3F* there appears to be more titania and silica eluted in the presence of (Tris-HCl) buffer at pH 9. This could indicate that a slight change in the pH could potentially alter the characteristic and the behaviour of silica and titania nanoparticles. This could be because as by mentioned *Jiang,* a change in pH alters the zeta potential of the nanoparticles which means the surface charge of the nanoparticles and hence interaction towards membrane channel would also be affected. Furthermore they demonstrated that when pH was brought close to the iso-electric point (I.E.P) of the nanoparticles; for titania (I.E.P) = 5.1 and for silica (I.E.P) = 2.5,[43, 44] they observed agglomeration of the nanoparticles.[45] This was also demonstrated by *Blasco and Picó,* whereby they observed silica nanoparticles agglomeration, under the view of scanning electron microscope (SEM) in the presence of artificial saliva at pH 2; which was close to (I.E.P) 2.5.[46]

For the experimental work physiological pH 7.5 that closely resembles human environmental conditions was selected, so to mimic human physiological environment and to understand how nanoparticles would behaviour in human physiological conditions. Interestingly *Xu,* their work on gold nanoparticles in phosphate buffer at different pH. They illustrated those gold nanoparticles in isotonic buffer at physiological pH 7.4 was the most stable without any aggregation being observed.[47]

On the contrary, enzymes (proteases and lipases) were also added in a selection of different buffers as a comparison. As shown in *fig 10.3H,* (Tris-HCl) at pH 7.5 with enzymes for 4hr. The type of buffer used does not appear to have much effect on the separation; all buffers used, produced well resolved void and sample peaks. However coffee creamer sample in (Tris-HCl) at pH 7.5 buffer appears to elute more sample material i.e. matrix and produced less ICP-MS signal noise compared with water, which could suggest that (Tris-HCl) buffer has higher active on the matrix than water. Furthermore as observed from the ICP-MS signal the titania nanoparticles and silica nanoparticles eluted together at 5-10mins, but less titania seem to be present in the void peak compared with without digestions as shown in *fig 10.3L*. This indicated that by enzymatic digestion, the enzymes could have potentially changed and altered the surrounding matrix environment and hence nanoparticles behaviour could well have been affected or could be that titania present in the matrix may have been digested.[1] Interestingly it was mentioned by *Wagner* that the presence of matrix plays an influential role on the behaviour of nanoparticles. In particular there have been reports that nanoparticles could be stabilised by the matrix.[23] On the otherhand examining the samples

without the presence of enzyme, silica nanoparticles eluted together with this time a lot more titania, which appears to be still present in the matrix between 10-25mins as shown in *fig 10.3K and 10.3L.* For the filtered sample less of the sample materials i.e. matrix can be seen. This could be as explained by *Kammer,* whereby some of the sample materials can be loss, due to the sample filtration process as shown in *fig 10.3I and fig 10.3J* lower signal in the silica and titania ICP-MS signal was observed.[15]

As illustrated by *Kammer* the composition and type of the carriers used will have a direct influence on both the nanoparticles adsorption behaviour towards the (FFF) membrane channel ultimately affecting the retention time of the fractograms peak hence the separation quality too.[15] It was highlighted that the use of just one type of carrier would not be sufficient if investigating optimisation. That's because the combination of the different chemical, pH and ionic strength composition that makes up the carriers could potentially change the electrostatic properties of the nanoparticles therefore affecting the interaction with (FFF) membrane channel and tubing.[15, 24] As demonstrated by *Whitley* her work on the optimisation of carriers in which she selected 18MΩcm^{-1} ultrapure water, she noted that the carrier reduced and minimised the possible interaction between the sample and the carrier solvent. This was an ideal situation as it prevented the carrier solvent from encouraging any form of sample aggregation, agglomeration and adsorption behaviour during the (FFF) separation stage.[33] This was demonstrated in *fig 10.5C,* well resolved void and sample peak with good symmetrical sample shaped peak obtained. In addition *Goenaga-Infante, H and co-workers,* their work on the characterisation of (TiO_2) in sunscreen, mentions the fact that using ultrapure water as a carrier suits (ICP-MS) instrumentation as it is well compatible with the instrument along with better stability for (Au) certified reference materials. Moreover they noted that using the same solvents for both sample and the (FFF) carriers minimises the possibility of nanoparticle changing, so the type of solvents used could play a key role too.[27]

For the experimental work three type of carriers were examined which were water, (Tris-HCl) and sodium dodecyl sulphate (SDS). As shown in *fig 10.4A, 10.4B and 10.4C*, for crude coffee creamer sample in the presence of water, (Tris-HCl) and (SDS) using water as the (FFF) carrier. Large void peak was observed and both the void as well as sample peaks were not resolved. In addition separation of the nanoparticles from the matrix was not observable. However with the filtered coffee creamer sample *fig 10.4D, 10.4E and 10.4F* using water as carrier. There were separation observed between the titania and silica nanoparticles early in the fractogram run between 5-10mins, thou not much sample materials i.e. matrix was present. This could be because of the filtration process altering and reducing the sample matrix hence affecting nanoparticle behaviour.[15] Or it could be the possibility that remaining samples were residing in membrane channel and tubing within the

(FFF) instrument from previous runs may have affected the sample run therefore producing different results for both the crude and filtered samples, this was mentioned by *Podzimek.* He proposed that after each sample run, (FFF) cross flow setting should be set back to zero for at least 5mins and allow the remaining samples to flush through to prevent this from occurring, but this would ultimately prolong analysis time.[48] On the otherhand *fig 10.4G, 10.4H and 10.4I*, crude coffee creamer sample present in water, (Tris-HCl) and (SDS) buffers but this time using (Tris-HCl) at pH 7.5 as the carrier. Both void and sample peaks were well resolved with separation between silica and titania nanoparticles was being observed. In addition as shown in *fig 10.4G* they were eluting separately, titania between 25-35mins and silica between 20-25mins. This was an indication that silica nanoparticles were separating from the matrix, with the matrix containing large amounts of titania. However this was surprisingly different to the result for crude sample in the presence of water, the titania nanoparticles had a much longer retention time eluting late in the fractogram run at around 20mins but this time titania eluted together with silica nanoparticles. As mentioned earlier this could be because of higher ionic strength solvents such as (Tris-HCl) changing the electrostatic properties of titania therefore the elution profile could also have changed.[42] Interestingly this was demonstrated by *Moon*, their work on the size characterisation of liposomes by Flow (FFF). They mentioned that the ionic strength of the carriers has a major influence on the efficiency of the separation of liposome. They noted that when comparing the (Tris-HCl) to (PBS) carriers, the fractograms retention time was much shorter in the presence of (Tris-HCl), due to the difference in the ionic strength of the two carriers.[49] In addition *Bolea* work on (Ag) nanoparticle also observed a much later fractogram peak for ultrapure water carrier compared with an earlier peak being observed when using 0.01% (SDS) carrier. They concluded that this could be again due to the carrier influencing the interaction to (FFF) membrane during the (AF^4) focusing/ relaxation steps, which changes the nanoparticle distribution within the channel.[34] Or it may even be as explained by *Ding and Pacek,* their work on hydrophilic silica nanoparticles and the effects of different types of surfactants at different pH ranges. They noted that surfactants such as (SDS) actually promoted de-agglomeration particularly at high pH levels. This could indicate that the coffee creamer sample in the presence of (SDS) buffers actually broken up the large matrix clusters, meaning titania elutes much earlier.[50] For the experimental work (SDS) carrier was examined however surfactant caused filtration pump to clog as a result unable to retrieve experimental data for this carrier. On the contrary there have been reports of (Tris-HCl) being used on nanoparticles extensively for aiding conjugation of functional groups modifying their surfaces. As mentioned by *Minteer,* protocols for conjugation of nanoparticles with DNA probe, they noted that (Tris-HCl) buffer appears to have a stabilising effect and also neutralises surface charges on the modified nanoparticles.[44] Moreover *Somma*

mentions the fact that (Tris-HCl) gives solutions a pH buffer capacity, which could be the reason as to why it has stabilising properties, as well as it being an ideal buffer that is not too harsh for nanoparticles.[51]

From these results obtained the overall conclusion was that with (Tris-HCl) carrier appears to offer better separation of silica and titania nanoparticles from the matrix. The results obtained suggest that titania and the silica are eluted separately. Compared to using water carrier where the nanoparticles eluted together from the matrix. This could be due (Tris-HCl) causing changes in the nanoparticle electrostatic interactions. On the contrary with water as buffer and carrier, matrix appears to be intact, but nanoparticles do appear to still elute from the (FFF).

8.23 Enzymatic digestion

As explained by *Blasco and Picó*, sample preparation of food samples before analysis are essential and necessary to ensure that the nanoparticles are isolated from the food matrix before characterisation of nanoparticle can be performed. This was because the matrix can cause potential interferences during characterisation of nanoparticles. Moreover nanoparticles could potentially change as the surrounding matrix environment changes because of sample preparation, thus preparation of samples must be done with care. As a result it is vital that any sample preparation processes carried out are recorded.[46] Ideally with sample preparation a compromise between reducing the sample matrix and preserving sample's representativeness should be achieved. There are several sample preparation techniques available depending on the sample type. With sample preparation the goal is to create a condition in which the nanoparticles and the surrounding matrices behave differently in order for separation to be achieved. Acidic digestion has been applied before extracting silica nanoparticles from a biological matrix, unfortunately this type approach will could potentially alter or even dissolve nanoparticles if conditions set are too harsh.[15, 23] Another potential problem with chemical digestion was the fact that some matrices may not respond. For example *Wagner, S* highlighted during their work on the quantitative analysis of engineered nanoparticles in food. They mentioned the fact that alkaline digestion for extracting (SiO_2) nanoparticles in tomato soup was not applicable as it had no effect on the food matrix. Furthermore they mentioned that enzymatic digestion was also used as well; one example that successfully worked was the extraction of silver (Ag) nanoparticles from meat matrix. There they used proteinase K to remove the matrix they reported no effects on (Ag) nanoparticles.[23]

In the experimental work enzymatic digestion was performed, a preferable method that was not too harsh on the nanoparticles but at the same time had the potential to remove the coffee creamer matrix. The presence of milk micelles (20-300nm) and fat micelles (1-2µm) in coffee creamer matrix could be potentially digested using a mixture of proteases and lipases.[19, 52, 53]

With the experiment prior to analysis, sample filtrations were performed. As reported by *Kammer,* 0.45µm filtration would allow sufficient removal of large milk micelle and fat from the sample matrix, in addition crude coffee creamer sample were also compared.[15] The duration of the digestion was also assessed, as a result a period of 4hrs and 24hrs digestions were performed. As previously mentioned the enzyme digestion for duration of 4hrs, both the titania and silica nanoparticles peaks were separated and were well resolved as shown in *fig 10.3G* and *fig 10.3H*, however a lot more sample materials were seen late in fractograms at 20-25mins, suggesting aggregation could have occurred or simply a lot of the sample material was present. Nevertheless this could indicate that enzymatic digestion could potentially change the surrounding matrix environment and hence nanoparticles behaviour was also being affected, which could be the reason behind the possible aggregation being observed.[1] Furthermore comparing 4hrs to the 24hrs enzyme digestion, this was shown in both fig *10.3H* and *fig 10.5B*. With 24hrs digestion both void and sample peaks were well separated and both of the titania and silica peaks eluted together, which can be observed late in the fractograms at around 20-25mins. With the 4hrs digestion, sample peak eluted much later between 25-30mins, which could suggest the nanoparticle behaved differently depending on the length of the digestion. In addition it was noticeable with the 4hrs digestion; a lot of titania was still present at 20-30mins. This could be due to more of the titania nanoparticle present in the sample matrix or that enzymatic digestion was insufficient. Moreover with the 24hrs digestion the eluted sample peak appears more symmetrical in its distribution and it also appears to display less noise which is an indicator of the quality of the separation.[15] Moreover as mentioned by *Tadjiki,* symmetry of the fractogram peak is related to particle aggregation. As a result the 24hrs digestion produced a better sample symmetry peak at 20-30mins compared to 4hrs digestion meaning nanoparticle aggregation was reduced in the 24hrs digestion. This could be an indication that nanoparticle aggregation reduces after pro-longed enzymatic digestion.[54] Or it could be as mentioned by *Arakawa* due to the injection flow rate affecting the overall shape of the fratogram peaks. They observed how protein separated by (AF^4) at high injection flow rate setting of 0.4ml/min from the initial low setting of 0.1ml/min produced much sharper and symmetrically shaped peaks.[55] Another reason could simply be because of the duration of the enzyme digestion acting on the coffee creamer matrix. It could suggest that longer the enzymatic digestion, hence more of the

coffee creamer matrix becomes digested. Or as mentioned by *Poda* with their work on (Ag) nanoparticle sizing using (FFF) it could be because of the size of nanoparticle. They reported that the noise of the fractogram signal increased as the (Ag) nanoparticle increased in size from 10nm to 80nm. They noted that due to the larger nanoparticle size it resulted in an increased noise in the (ICP-MS) signal because of larger amounts of particle introduced into the (ICP-MS) detector.[30] So it could be that larger nanoparticles were present in the 4hrs digestion compared to 24hrs digestion, as a result nanoparticle sizes changed due to the length of digestion.

However the effectiveness of the enzymatic digestion may be subjected to the enzymes working conditions in which they are set at. Because of this the optimal working condition of the enzymes could have a major influence on the digestion of the coffee creamer matrix. *Rekha* reported that the optimal working conditions for lipase from *candida rugosa* was at pH 6.0 with temperature of 32°C and five day incubation period produced maximum lipase activity which they obtained an activity of 57.25U/ml.[56] Moreover *Bellucci* highlighted how lipase activities differ in the presence of different buffers and at different pH ranges. For instance maximum activity was achieved using (Tris-HCl) at pH 5.4, compared to citrate and phosphate buffer at pH 6 and 6.7 repectively.[41] On the otherhand optimisation studies on the protease production from *streptomyces griseus* was demonstrated by *El-Gammal.* They found that maximum activity was achieved at pH 8, with temperature at 30°C after a period of six days incubation.[57]

Comparing crude and the filtered coffee digested coffee creamer sample *fig 10.5C* and *fig 10.5D*. The fractogram obtained indicated that the filtration process reduced the titania and silica nanoparticle which suggests that the larger particles such as milk micelles and fat micelles have been reduced or even removed.[11, 15] Furthermore looking at no digestion as shown in *fig 10.3K* and *10.3L* samples without enzymatic digestion, titania peak had a slight tailing, sign that aggregation was present. Furthermore as mentioned earlier a lot more titania was observed early in the peak fractograms at 10mins. In addition a lot more aggregated titania nanoparticles were present between 20-25mins and eluted much earlier at around 10mins, compared with enzyme digested in fig *10.5A,* whereby little titania eluted at 10mins and much was eluted late in the fractograms at around 20-25mins. This indicated that without any enzymatic digestion, titania nanoparticles was still readily present in the coffee creamer matrix as enzyme was not present to break down titania. Interestingly work carried out by *McKinnon* involved using (EDTA) to fragment and disperse milk sample matrix. They illustrated that using 50mM (EDTA) allowed large milk micelles (>100nm) and small milk micelles (10-40nm) to disintegrate as shown from the fractogram obtained. The

sample with (EDTA) added, the peak for the large particle micelles was almost non-existent. This could be an alternative solution to filtration process in reducing the coffee creamer complex matrix.[58]

Overall enzyme digestion has the potential to digest the complex matrix reducing its complexity so that nanoparticles can be successfully extracted, but there was no clear indication whether nanoparticle had changed or not. As a result (TEM) work could potentially reveal this.

8.3 Quantitative analysis - Silica injected mass and silica (%) percentage recovery

8.31 Crude and filtered coffee creamer

The quantification of silica nanoparticles present in coffee creamer sample was performed by using post – FFF channel calibration as adapted from *Nischwitz, V.* and was demonstrated by *Goenaga-Infante, H.* and co-workers with their work on characterisation of (TiO_2) in sunscreens.[26, 27] The total silica injected into (FFF-ICP-MS) was compared to that of total silica by direct injection by (ICP-MS) to enable mass balance calculation; to assess the potential loss of silica nanoparticles due to sticking onto (FFF) membrane and tubing.[5] The advantages of this approach is that it does not require calibration to be directly injected into the (FFF) as solution can be taken up through (ICP-MS) nebuliser and the simplistic method whereby calibration can be added in between or at the end of sample runs with ease is an added advantage. As a result information concerning possible nanoparticle interaction along (FFF) tubing and membrane channel can be obtained by assessing the recovery rates.[30]

For the experimental work both crude and filtered coffee creamer sample silica recovery were examined as shown in appendix part B *table 10.1A*. The silica (%) recovery obtained on both *12 June* and *14 June* was 19.8% and 22.3% respectively under direct injection. Very similar recoveries were obtained over those two days. This suggests that even thou no crossflow was applied the silica nanoparticle interaction with (FFF) membrane and tubing was still occurring due to nanoparticle sticking. As noted by *Mitrano,* one of the limitations in performing quantitative analysis using (FFF-ICP-MS) was the potential of nanoparticles interaction with the (FFF) membrane channels and tubing, influencing the quality of the separation as well as the recovery rates.[28] Moreover there are possibilities that nanoparticles could also be lose at the (ICP-MS) nebuliser and spray chamber too.[27, 28] Furthermore (%) recovery was reduced to 12.8% as coffee creamer sample eluted from the

(FFF) under the influence of crossflow 1ml/min. This verifies that interaction with the membrane channel was occurring because at higher cross flow, the force running perpendicular towards the channel forces the nanoparticles to stick even closer to the membrane walls, preventing sample from eluting out, thus a potential loss of silica nanoparticle was occurring through the permeable membrane walls as highlighted by *Qureshi and Kok.*[35] In addition the charge on the surface of the nanoparticle could play a role in the interaction as reported by *Yu*, bare silica nanoparticles that possesses negative surface charges. They could potentially interact with the regenerated surface that possesses excessive charge density, resulting in nanoparticle-membrane interaction.[15, 59]

On the contrary the injected silica mass obtained was much higher for crude sample at 373.7µg/g compared with the filtered crude sample at 47.9µg/g. This verifies that the filtered coffee creamer sample was potentially losing silica nanoparticles during filtration process. *Kammer* highlighted the fact that filtration process could potentially change the nanoparticles due to some being embedded into milk micelles matrices and not being able to pass through the filtration pore size. Meaning the amount of nanoparticles present would have changes in addition the behaviour could change due to matrix alteration therefore the accuracy of the silica quantification could have been affected.[1, 15]

8.32 In the presence of different buffers and carriers at specific pH

As shown in *table 10.1B* comparing the silica (%) recoveries rate for the crude coffee creamer sample in a selection of different buffers and carriers. Crude sample in (SDS) using water carrier produced excellent silica (%) recoveries at 90.8%. This could be as mentioned earlier by *Ding and Pacek,* surfactants such as (SDS) actually promoted de-agglomeration particularly at high pH levels because of electrostatic mechanisms, as a result dispersing nanoparticle which provides better stability during (FFF) elution.[50] In addition as illustrated by *Bolea* surfactants added to the sample and also in carrier solvents showed that they both had direct influence on the (%) recovery. For their studies on (Ag) nanoparticles recovery rates; 0.01% (SDS) at pH 8 obtained the best recovery at 98+-1% for 10nm size particle and for larger particle at 80nm (%) recovery was 106+-6%.[34] Furthermore good (%) recoveries for sample in (Tris-HCl) using water carrier and sample in water using (Tris-HCl) carrier at 64.5% and 77.7% were obtained respectively. (Tris-HCl) buffer appears to promote stability towards the nanoparticle due to high recovery rate obtained. Interestingly experimental studies demonstrated by *Langer,* on the development of a potential drug carrier through

exploiting nanoparticle cross-linking to human serum albumin (HSA). They demonstrated that (HSA) nanoparticle in the presence of 0.2M (Tris-HCl) buffer at pH 8; nanoparticle did not precipitate at all with (HSA). They suggest that this could be due to the (Tris-HCl) molecule amino group interfering with cross-links of the (HSA) matrix. As a result there is a possibility that (Tris-HCl) buffer could be interacting in a similar way with coffee creamer matrix, preventing aggregation.[60]

Overall good recovery rates were obtained for both (Tris-HCl) and (SDS) could be because of the ionic strength of the solvent changing the nanoparticle interaction or minimising the aggregation of nanoparticle.

On the otherhand low recoveries were observed for both (Tris-HCl) and (SDS) buffer in (Tris-HCl) carrier. *Bolea* explained that with low recoveries it could be because of the interaction between the ionic shield strength of the carrier and the membrane channel.[34] As demonstrated by *Bagwe,* their work on modified silica aggregation studies illustrated that the zeta potential, the surface charge present around the nanoparticle when reduced to near zero values resulted in the repulsion interaction to decrease.[61] Moreover the type of buffer at different pH could also have a direct influence on the nanoparticle surfaces charges. For instance *Bagwe*, noted that the (Tris-HCl) at pH 7.4 used in their experiment, the buffer appeared to have affected the amine group on the modified silica nanoparticles to become positive, meaning that the surface charge on nanoparticles changed. However some of the unaffected nanoparticles were still negatively charged, thus aggregation occurred between the two. Furthermore the (FFF) membrane channel; regenerated cellulose is slightly negative charged, as a result nanoparticle adsorption onto membrane could be increased.

Overall the change in the nanoparticle surface due to the choice of buffers used and the reduced repulsive forces asserted could have potentially lead to the decreased recoveries observed.[34, 61]

8.33 Spiking silica nanoparticles in coffee creamer sample

The characterisation of silica nanoparticles standards will be important for the future work on isotopic dilution mass spectrometry (IDMS) methodology as well as potential development of certified reference materials. With (IDMS) the aim is to improve the accuracy of silica quantification in complex matrices. The advantages of (IDMS) were highlighted by *Diemer and Heumann* the problem with quantification of silica would be the fact that during

sample preparation stage, silica could be loss which could have potentially affected the quantification accuracy. As a result this would not necessarily give a good representation of the silica actually present in the sample. However with (IDMS) any loss of silica nanoparticle would not directly affect the nanoparticle quantification, so the accuracy would not be compromised.[62, 63] As a result the selection of spike nanoparticles to use for (IDMS) will be important.

The experimental work investigated alternative silica nanoparticles which could offer better compatibility and stability silica nanoparticles in the presence of the coffee creamer sample for use as potential spikes. As shown in *table 10.1C* both Strober and ($SiOAR_4$) silica nanoparticles were compared in the presence of coffee creamer sample using (Tris-HCl) carrier. The silica (%) recoveries obtained for both nanoparticles were poor, obtaining results between 23-26%. In addition in the presence of crude coffee creamer sample and crude with enzymes digestion, silica (%) recoveries were average, obtaining results between 41-56%. It could be possible that (Tris-HCl) carrier does not suit either silica standards. Or as mentioned by *Goenaga-Infante, H.* and co-workers due to the high crossflow setting at 3ml/min, this actually prevented silica from reaching the (ICP-MS) detector as they are forced closer to membrane channel walls. In addition it could also be due to the influence of coffee creamer matrix affecting the silica standards and thus potentially promoting aggregation.[27] This was verified by the (MALS) fractograms as observed in *fig 10.6A* in appendix part A, where the Strober eluted late in the fractogram at around 30-35mins with noticeable large particles observed in (MALS), which could be the aggregatation. On the otherhand ($SiOAR_4$) nanoparticles appears to show earlier elution peak between 15-20mins shown in *fig 10.6C*, which suggests that it could be a better candidate as no aggregation was observable in the fractogram.

To assess the suitability of the two standards a direct comparison between the two standards were performed using water carrier as shown in *table 10.1D.* Both silica nanoparticles gave good silica (%) recoveries between 81-88%. The recovery rate obtained for ($SiOAR_4$) was slightly higher than for the Strober. It was reported by *Bagwe, that* adding different functional groups onto nanoparticles reduced nanoparticle non-specific binding and aggregation.[61] In the case of ($SiOAR_4$), the arginine functional group offers better stability for the silica nanoparticle. Interestingly as reported by *Zhang, Z* his studies on the development of nanoparticles for the purpose of biomedical imaging. He noted that (Au) nanoparticles with functionalised rich arginine peptide were highly stable in 0.6 M (NaCl) at pH 6. He explained that this could be due to the high basicity of the guanidinium functional group found in arginine.[64]

Repeated injections of silica were performed to assess the reproducibility of the results. From the results obtained both silica standards shown good repeatability at 2.55% and 6.86% respectively giving a coefficient variance of less than 10%.

As shown in *fig 10.5E in* appendix part A, the ($SiOAR_4$) silica standard elution profile produced a small fractogram peak between 5-10mins can also be seen in the (ICP-MS) signal, slight aggregation could be observed at 25mins. The approximate size of the silica standard obtained was a radius of 16nm and molecular mass of $4.45x10^4$ Da based on the (FFF) software calculation. From *table 10.1E* looking at comparing the ($SiOAR_4$); that was spiked in coffee creamer sample and in water. The (%) recovery rate for spiked in coffee creamer was slightly lower than at 72.6% than for the one spiked in water only at 92.6%, under crossflow 1ml/min. This would suggest that the silica standard in presence of crude coffee creamer sample was less stable. As mentioned by *Goenaga-Infante, H.* and co-workers the nanoparticles could be influenced by the surrounding matrix environment therefore stability of the standards could have been affected.[27] At a higher crossflow setting the (%) recoveries obtained were almost halved. This proves that at high crossflow settings nanoparticle are forced closer to membrane channel as a result loses in silica nanoparticles were observed.[35]

From the results obtained, both silica nanoparticles at crossflow 1ml/min and 3ml/min produced good repeatability at 12.4% and 13.7% respectively, giving a coefficient variance of less than 10%.

8.34 Comparison between NanoChop and Klebosol silica nanoparticles

As shown in *fig 10.7A* and *fig 10.7C* in appendix part A, the elution profile for both silica nanoparticles produced a good fractogram peak. This can also be seen in the (ICP-MS) signal between 5-10mins, a very similar profile for ($SiOAR_4$) was also obtained. However slight aggregation can be observed at around 30mins. The approximate size of the NanoChop silica obtained was a radius of 20nm and molecular mass of 10^7 Da based on the (FFF) software calculation. The size of the aggregated NanoChop silica was a radius of 53nm and molecular mass of 10^{12} Da. On the otherhand the approximate size of the Klebosol silica obtained was a radius of 50nm and molecular mass of 10^7 Da. The size of the aggregates Klebosol silica was a radius of 90nm and molecular mass of 10^{12} Da. From *table 10.1F* examining the (%) silica recoveries of between NanoChop and Klebosol, Klebosol had a higher (%) recovery at 94.5% compared with NanoChop which had a (%) recovery of 63.4%. This could be due to the presence of stabiliser cations on Klebosol nanoparticles

such as Sodium (Na), Potassium (K), Ammonium (NH_4) and Aluminium (Al) that exerts a repulsion force between the particles resulting in better (%) recovery obtained.[65] In addition with crossflow set at 1ml/min, NanoChop recovery rate was decreased. This again was due to the stronger crossflow force acting on the nanoparticles.[35]

In addition the reproducibility of the result was good, for both the NanoChop and Klebosol, the coefficient variance obtained were 4.00% and 0.17%. Thou Klebesol appear to have a better reproducibility, could be because higher recovery rates was obtained.

Overall comparing the Klebsol and the Strober with both Strober and ($SiOAR_4$) standards, the (%) recovery for both were good and showed very similar (%) recoveries.

Investigation into the use of (Tris-HCl) as a buffer as well as carrier for NanoChop was performed as shown from *fig 10.7A in* appendix part A, the elution profile acquired was very similar to the one carried out in water. Although this time the fractogram peak between 5-10mins and 20-30mins did show some aggregation were present. From *table 10.1G*, the silica (%) recovery rate, this time in the presence of (Tris-HCl) was excellent at 93%, compared with in *table 10.1B*. This could as mentioned by *Bolea* whilst they investigating recovery rates for their work on (Ag) nanoparticles. Due to repeated injections of the sample, that helped to condition the (FFF) membrane channel. As a result this reduced the adsorption of nanoparticles interacting with the (FFF) membrane and tubing, hence minimising the potential loss of silica.[34]

Furthermore the reproducibility of the run was good; the calculated coefficient variance was 0.68%. In addition the total silica counts present in the coffee creamer sample with (Tris-HCl) buffer was calculated to be 812µg/g using direct injection by (ICP-MS) analysis.

Overall conclusion for the characterisation of silica standards was that both Strober and Klebosol nanoparticles were both potential candidates for spike work, for the development of (IDMS). In addition apart from using water as buffer as well as carrier, (Tris-HCl) is also a substitute solvent. Furthermore the results collected were encouraging and good silica (%) recoveries were obtained. However it is important to bear in mind that recovery rates can be influenced by various analytical parameters. *Dubascoux* provides a good summary account to the problem; they included a list of the problems that could affect the results. They included the following issues:[66]

- the type of membrane channel
- membrane channel size dimension,
- ionic strength of carrier and its composition,
- presence of large void peak, distorting quantification analysis
- direct injection under no crossflow, is it true that no dissolved sample remains in the channel at all.

8.4 Quantitative analysis – Total silica counts by microwave digestion

8.41 Total silica counts

Total silica counts in samples were performed using direct (ICP-MS) analysis assisted by microwave digestion adapted from a previously performed protocol by *Goenaga-Infante, H.* and co-workers.[27] Total silica counts can be difficult to achieve; therefore total digestion using microwave technique would allow accurate quantification measurements to be obtained compared to just using enzymatic digestion. This was due to the complexity of the matrix environment in which silica could potentially be embedded into or aggregated with larger matrix material.[67] Hydrofluoric acid (HF) was used to digest the matrix to give individual silica nanoparticles. (HF) has been previously used to remove silica particles from silicone wafers (found in electrical circuit boards) as illustrated by *Bachman*.[68] As shown in *table 10.2A, B and C* in appendix part B, total silica counts in the presence of (Ge) (Rh) and (Y) internal standards. Looking at the Si/ Ge result obtained the average Si^{28} and Si^{29} counts for the digested crude coffee creamer sample were 748.6µg/g and 762.8µg/g respectively. For Si/ (Rh) average Si^{28} and Si^{29} counts were 706.7µg/g and 720.1µg/g. For Si/ Y average average Si^{28} and Si^{29} counts were 748.6µg/g and 762.8µg/g. Both crude coffee creamer and (zolt 3) samples gave good reproducibility and coefficient variances were below 10%. However Si/ Ge and Si/ Y obtained slightly lower CV (%) results compared with Ge/ Rh. The type of internal standards to use for total digestion is important because ICP-MS solvents can have an effect on the (%) recovery rates as well as silica extraction. As highlighted by *Pruszkowski,* (Ge) has been commonly used as an internal standard for ICP-MS analysis.[69] As a result the results obtained do show that (Ge) as well as (Y) internal standards are both very well suited for total digestion.

However looking at (zolt 1) and QC digested samples, the CV (%) results obtained was more than 10% which was not within the accepted range. In addition looking at the (%) recovery rate as shown in *table 10.1D, E and F*, both QC 2 and digested blank were higher

than expected this could be due to the high silica background obtained. There could be several experimental factors which could have potentially affected the results, this included:

- Firstly the wrong solutions could have been used.
- Secondly glassware could have been contaminated with silica content.
- Thirdly old reagents including (HF) could have been used.
- Finally human errors such as typos, pipetting, weighing could have made an impact.

However looking at the calibration point (%) recoveries rates they were all within the 100% target, this means that the method performed was valid. As demonstrated by *Pruszkowski* with her studies on total Selenium (Se) counts in serum. She explained that the accuracy of the quantitative analysis depended on the sample preparation and calibration. She reported that high selenium background obtained was due to incomplete microwave digestion of carbon sample matrix.[69] In addition other limitations with the method included:[69]

- the requirement for large dilution of samples;
- high acid concentration used for final solutions
- long sample preparation time unless there are alternatives methods avalible.
- Safety aspects of using concentrated (HF)

On the otherhand *Poda* mentions that aggressive microwave digestion for total count analysis is usually the preferred method for achieving successful total digestion of the sample matrix, however the method could potentially change the nanoparticle shape and size.[30]

Meanwhile looking at the (%) extraction efficiencies shown in *table 10.2A, B and C*. Crude coffee creamer sample appears to have much lower (%) extraction efficiencies compared to filtered coffee creamer. This could be due to complex matrix being reduced by filtration process, as a result reducing the silica nanoparticle interaction with the matrix which in turn increases nanoparticle interaction with (FFF) membrane channel and tubings.[15] However both (zolt 1) and (zolt 3) gave good extraction efficiencies of between 83-96%. This could suggest that both silica nanoparticles are relatively stable with minimal interaction, without the presence of sample matrix. However extraction could be improved as noted by *Goenaga-Infante, H.* and co-workers, nanoparticle suspension should be vigorously shaken and sonicated prior to analysis to ensure nanoparticles are well dispersed and uniform as nanoparticle distribution this could have affected the (%) extraction efficiency results.[27]

8.5 Qualitative analysis - Transmission Electron Microscope (TEM)

8.51 Crude coffee creamer in (Tris-HCl) buffer

Looking back at the previous results collected for both the (MALS) fractograms and (ICP-MS) signals. For the crude coffee creamer in the presence of water using (FFF) water carrier, as shown in *fig 10.4A* in appendix part A. Both the silica and titania nanoparticles eluted together from the matrix, quite early between 5-10mins. On the otherhand compared with the crude coffee creamer in the presence of (Tris-HCl) at pH 7.5 using (Tris-HCl) carrier, as shown in *fig 10.8B* in appendix part A. Silica eluted slightly earlier at 20-25mins than titania nanoparticles at 25-30mins. This suggested that separation between silica and titania nanoparticles from the matrix could have occurred. As a result the use of (TEM) imagery would verify whether this phenomenon had indeed occurred, by collecting (FFF) fraction between the time points of 22-26mins for silica and 28-31mins for titania. As illustrated the (TEM) image as shown in *fig 10.1A* in appendix part C, crude coffee creamer in (Tris-HCl) buffer using (Tris-HCl) carrier, silica nanoparticles in the size range of 5-7nm can be observed, these would be classified as nano-sized particles. In addition there were also larger particles observed in the size range of 20-30nm which are possibly titania nanoparticles that have aggregated in the presence of the (Tris-HCl) buffer. As a result this indicates that (Tris-HCl) buffer did in fact allow the separation of the silica nanoparticle between 20-30mins from the titania between 25-35mins which was present in the matrix, as shown in *fig 10.8B* in appendix part A. Interestingly comparing coffee creamer with water which *Weigel* illustrated under (TEM) imaging. The coffee creamer with water revealed much larger silica nanoparticle structures between size range of 60-160nm. This could mean that extraction of silica nanoparticle from coffee creamer matrix would not be viable as aggregation was present.[23] Moreover as shown in *fig 10.1B* in appendix part C, the later fraction collected between 28-31mins, titania nanoparticles 3nm can be observed as well as larger titania at 30-50nm. As illustrated by *Zhang,* their work on determining the role of (Tris-HCl) on melamine-modified gold nanoparticles, they noted that with 10mM (Tris-HCl) a reduction in the aggregation of nanoparticles was seen. They believed that this could be due to the (Tris-HCl) disrupts the nanoparticle electrostatic repulsion mechanism, which are important for the process of aggregation to work.[70] Interestingly as mentioned by *Somma*, his work on the extraction and purification of DNA from genetically modified crops. He highlighted the fact that (Tris-HCl) was used as an extraction buffer for the isolation of DNA from plant samples. He noted that (Tris-HCl) with (EDTA) and (CTAB) dissolved the surrounding fatty lipids of cellular membranes which enabled the intact (DNA) to be released from the samples.[51]

Furthermore (TEM) imaging was also performed for both Klebosol and NanoChop silica nanoparticles. As shown in *fig 10.2A* and *10.2B* in appendix part C, in contrast to the coffee creamer sample silica nanoparticles are uniform and spherical in shape and size. Klebosol silica nanoparticles size range was 60nm, in comparison to the MALS calculated radius of 60nm, which were both identical. On the otherhand NanoChop standards had a varying size range of between 40-90nm, in comparison to the MALS calculated radius of 50nm, which were very similar. As result it appears that Klebosol silica nanoparticles had a better consistency in its shape and size compared with NanoChop silica nanoparticles. This could be the reason as to why the fractograms peak shown in *fig 10.7A* in appendix part A was actually slightly wider compared with *fig 10.7C*.

9. Conclusion and Future Works

The choice of (FFF) parameters as well as sample preparation play an important and influential role in the separation of silica nanoparticle and titania from its complex matrix. By comparing and contrasting earlier methods by developed (LGC). Optimal (FFF) parameters were determined and the optimal crossflow, injection time and field programme settings were selected for the quantification of silica nanoparticle. Overall a high crossflow setting at 3ml/min appears to allow better separation of the nanoparticle from its complex food matrix. A high injection time would allow low void peak to be obtained, meaning minimal loss of sample would occur. In addition a linear decay field setting allows analysis runtime to be shortened without compromising the separation of the sample.

Furthermore the use of (Tris-HCl) as a buffer and carrier for the crude coffee creamer has shown great potential for achieving silica nanoparticles separation from its complex matrices that contains large amounts of titania nanoparticles. Moreover (TEM) imagery verified that silica nanoparticle was indeed extracted and isolated from the coffee creamer matrix and importantly the nanoparticle was still present in the nano-size range of less than 100nm. Both silica and titania in its native primary form as well as aggregated form were observable under (TEM).

The recovery rates were assessed to determine potential loses of silica nanoparticle due to sticking onto (FFF) membrane channel and tubing. Good silica (%) recovery rates were obtained for crude sample in (Tris-HCl) was between 93-100%. Total silica counts present in the coffee creamer sample was analysed by direct (ICP-MS) analysis and gave a result of 812µg/g. However during the sample preparation process and the enzymatic digestion possible changes to the nanoparticle could have occurred. As a result there could

be a possibility that nanoparticles could have been lost during these processes, therefore affecting the accuracy of the silica quantification. For the future work (IDMS) will be carried out for the characterisation of nanoparticles in complex matrices, by using chosen silica nanoparticle spikes. The experimental work on the characterisation of silica nanoparticles revealed that both Klebosol and NanoChop nanoparticle are potential spike candidates. Spiking nanoparticles into crude coffee creamer sample would allow better accuracy in the quantification of silica present in the sample with complex matrices and the loss of silica during both the sample and digestion processes would be eliminated.

Citation

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