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Nanoparticle identification using single particle ICP-ToF-MS acquisition coupled to cluster analysis. From engineered to natural nanoparticles

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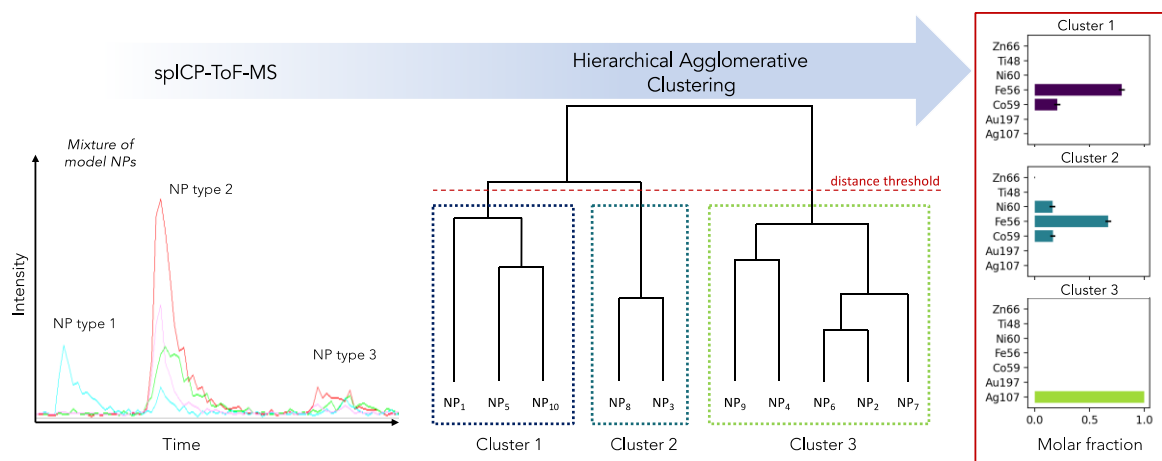
Abstract

In this study, mono-, bi-, and tri-metallic engineered nanoparticles (ENPs) are first analysed using an ICP-ToF-MS in single particle mode. The method is also used to characterize natural NPs (NNPs) (*i.e.* oxide and clay minerals). We provide here the elemental composition and mass distribution of these different NPs. For the first-time using spICP-ToF-MS, the presence of Ti- and Si-NPs as well as that of a second population with a distinct Si-Al ratio are highlighted in kaolinite. Finally, for mixtures of the different original NPs, an automated post-processing approach based on hierarchical agglomerative clustering (HAC) is applied. The elemental signature of the different clusters is in agreement with the results of previous characterizations. This illustrates the applicability of coupling spICP-ToF-MS with HAC to determine elemental fingerprints of NPs and to differentiate one from the other when mixed. The described methodology could be a starting point to simplify the analysis and make reliable interpretations of unknown NP populations and thereby more accurately understand NP populations in natural systems. However, as illustrated, thorough evaluation of the data must be performed in order to avoid misclassification of the NPs in future studies.

Keywords

engineered and natural nanoparticles, elemental signature, spICP-Time of Flight-MS, hierarchical agglomerative clustering

Graphical Abstract



30

31 Introduction

32 A better qualification and quantification of the anthropogenic impact coming from
 33 nanoparticles (NPs) release in the environment is linked to a better identification of the NP's origin.
 34 Several studies have shown that much of this can be achieved using the NP's elemental composition
 35 (*i.e.* fingerprint) ¹⁻³. However, the composition of engineered nanoparticles (ENPs) (*i.e.* purposely
 36 manufactured for specific purposes) can be quite similar to incidental (INPs) (*i.e.* by-products of
 37 human activities) and natural nanoparticles (NNPs) (*i.e.* produced by natural processes). Indeed, the
 38 production of ENPs requires elements present in soils and/or sediments from where some INPs and
 39 most NNPs come. Nevertheless, ENPs are produced for a specific purpose, for which they must have
 40 special properties, and therefore their elemental composition could differ from that of NNPs.
 41 Nevertheless, an identification of individual or group of NPs can be particularly challenging.

42 Single-particle inductively coupled plasma mass spectrometry (*i.e.* spICP-MS) is a technique
 43 which is able to not only investigate the particle number concentration and size (or mass), but also the
 44 elemental composition of individual nanoparticles ⁴⁻⁸. Briefly, NPs introduced into the plasma are
 45 ionized and generated ions are successively extracted through the interface, separated according to
 46 their m/z , collected at the detector and the signal is recorded by a computer at high frequency. The
 47 resulting signal of individual NP is then converted to a mass using an external calibration and the
 48 transport efficiency ⁹. Typically, the signal of a single NP event usually lasts between 200 and 1200
 49 μ s. Thus, a critical point for nanoparticle analysis/identification is the ability to monitor several
 50 elements/isotopes in an individual particle in this timeframe. Using a sector field (SF)-MS in single
 51 particle mode would allow very low detection limits (*i.e.* $D_{min} \leq 10$ nm for single element NP) ¹⁰.
 52 However, the single detector with milliseconds dwell-times (*i.e.* $t_d \geq 1$ ms) combined with the
 53 relatively slow rate of change of the magnetic field (few tens of milliseconds) to switch from one mass
 54 to charge ratio (m/z) to another prevents the possibility of monitoring multiple elements in an
 55 individual NP. Hirata *et al.* (2020) ¹¹, proposed spICP-MS analysis with a multi-collector (MC)-ICP-
 56 MS for Re/Os (¹⁸⁵Re/¹⁸⁸Os and ¹⁸⁷Os/¹⁸⁸Os) ratios on NPs produced through a laser-ablation system.
 57 Such a mass spectrometer is equipped with several detectors that allow the measurement of multiple

58 isotopes simultaneously. In the cited study, thanks to four high-time resolution ion counters, isotope
59 signals were recorded at very high acquisition rates (*i.e.* $t_d = 10 \mu\text{s}$). However, a MC-ICP-MS is not
60 adapted for multiple element analysis as it can only target a narrow range of m/z in one data
61 acquisition (*i.e.* Re/Os in the cited study). Recently, although quadrupole (Q)-MS have only one
62 detector, manufacturers increased the peak-jumping and acquisition rates (*i.e.* $t_d \leq 50 \mu\text{s}$)¹². These
63 improvements allowed better spICP-Q-MS time resolution for NP events and monitoring multiple
64 elements in each particle became possible. Although settling-times (*i.e.* time separating the
65 measurement of two consecutive masses) are relatively short (*i.e.* $\sim 50 \mu\text{s}$), the monitoring is limited to
66 two targeted elements if each NP event is to remain well resolved¹³.

67 Compared to the above, a time of flight (ToF)-MS does not use a magnetic, electrostatic, or
68 RF field to disperse or filter ions for individual m/z ion detection but instead uses a time discrimination
69 process to resolve ions of different mass¹⁴. Moreover, the short spectral-acquisition time presents one
70 of the major advantages of the time-of-flight mass analyzer as it enables the monitoring of all m/z ions
71 in the mass spectrum (quasi-)simultaneously ($\sim 2\mu\text{s}$ packets of ions sampled every 25-30 μs with 3-10
72 spectra summed and stored to disk continuously)¹⁴. Undeniably, an ICP-ToF-MS continuously
73 collecting the multi-ion beam at this high rate with no interruption allows:

74 i/ deconvolution of NP signals

75 ii/ collection of all NP signals without loss

76 iii/ reduction in the incidence of multiple NP being measured in the same acquisition period

77 iv/ determination of the (multi)-elemental composition of individual NPs

78 Thus, the identification of nanoparticles (NPs) in natural complex matrices (*i.e.* environmental,
79 geological, biological...) should be facilitated as the information on each measured NP is much more
80 thorough.

81 Nevertheless, at least one issue still needs to be addressed since the large amount of
82 data/information makes its interpretation very challenging. Several studies have used single-particle
83 ICP-ToF-MS for the characterization of NPs in complex/environmental matrices¹⁵⁻¹⁹. Praetorius *et al.*
84 (2017)¹⁵ were the first to use machine learning by means of a supervised gradient decision tree
85 boosting classification after spICP-ToF-MS. Then, Mehrabi *et al.* (2021)²⁰ and Baalousha *et al.*
86 (2021)²¹ combined time-of-flight measurement with another type of unsupervised machine learning
87 algorithm: the hierarchical agglomerative clustering (HAC). Thanks to the ToF-MS, they were able to
88 analyze metal and metalloid-containing NPs in wastewater samples and soil extractions and determine
89 their (multi)-elemental composition. For the first time, the use of the HAC helped them to
90 preliminarily classify the NPs and hypothesize their natural or anthropogenic origin. Although
91 innovative and very promising, the lack of information (*i.e.* composition, transformation) about the
92 NPs potentially present in these artificial and geological reservoirs limited the interpretation of the
93 provided data. To go further and more robustly determine their origin, both studies mentioned the need
94 to have more knowledge (*i.e.* data) about original NPs (*i.e.* engineered and natural).

95 Thus, to undertake the challenge of the identification of NP origin, this study had two
96 objectives: i/ illustrate the applicability of coupling spICP-ToF-MS at high acquisition rate to an
97 automated post-processing approach based on hierarchical agglomerative clustering for NP population
98 identification
99 ii/ provide information for future studies (*i.e.* dealing with the question of the origin and the
100 transformation of NPs in the environment).
101 These two objectives were completed by analyzing/characterizing NPs of known composition either
102 engineered or analogues of natural NPs (*i.e.* alumina and clay minerals).
103

104 **Material & Methods**

105 Chemicals

106 Ultrapure water (UPW; resistivity $\approx 18.2 \text{ M}\Omega \text{ cm}^{-1}$) from a Millipore Integral 5 (Millipore,
107 USA) was used throughout the work for dilutions. Mono-elemental standards (Au, Ag, Al, Si, Ti, Fe,
108 Zn, Co, Mg, Ca, La, Ce, Zr) at 1000 mg L^{-1} were obtained from SCP Science (Canada). Normapure
109 nitric acid (VWR, USA) was purified in a sub-boiler DST 1000 (Savillex, USA).

110 Spherical and citrate capped Au-NPs from British Biocell International (UK) with a certified
111 nominal diameter of 40, 60 and 80 nm were used for the determination of the sample transport rate.

112 For the proof-of-concept experiments, different types of NPs were used:

- 113 - Mono-metallic spherical and citrate capped Au- and Ag-NPs from BBI Solutions (UK)
114 with a certified nominal diameter of $79 \pm 6 \text{ nm}$ and $80 \pm 7 \text{ nm}$, respectively.
- 115 - Bi-metallic spherical and citrate capped core-shell Au-Ag-NPs from Nanocomposix
116 (USA) with a certified nominal diameter of $59 \pm 6 \text{ nm}$ and $79 \pm 9 \text{ nm}$ with a gold core
117 diameter of $30 \pm 3 \text{ nm}$ and $51 \pm 6 \text{ nm}$, respectively. For both NPs, based on certificates,
118 the respective calculated thickness of the Ag shell is 15 nm and 17 nm. To facilitate the
119 reading, they are referred to as 60 and 80 nm Ag@Au-NPs.
- 120 - Tri-metallic Nickel, Cobalt, Iron and Zinc, Cobalt, Iron oxide nanopowders, respectively
121 named FeCoNi and FeCoZn, from US Research Nanomaterials (USA) both with a
122 nominal diameter of 40 nm.

123 The theoretical molar fractions of all particles are displayed in Table 1.

124 As analogue of NNPs, two clay minerals, a kaolinite – *KGa-2* – and a Texas montmorillonite
125 – *STx-1b* –, were purchased from the Clay Mineral Society (CMS). Their composition and molar
126 fraction derived from the structure given on the CMS website
127 (http://www.clays.org/sourceclays_data.html) are displayed in the Table 1. For instance, given the
128 structure of STx-1b (*i.e.* $(\text{Ca}_{0.27} \text{Na}_{0.04} \text{K}_{0.01})[\text{Al}_{2.41} \text{Fe(III)}_{0.09} \text{Mn}_{\text{tr}} \text{Mg}_{0.71} \text{Ti}_{0.03}][\text{Si}_{8.00}]\text{O}_{20}(\text{OH})_4$) and
129 taking the major elements (*i.e.* Al, Si, Ti, Fe, Mg and Ca), the theoretical molar fraction of Al is
130 $2.41/(2.41+8.00+0.03+0.09+0.71+0.27) = 0.21$. Note that for model NNPs, the so-called theoretical

131 molar fraction is actually the molar fraction of the bulk material. However, the term “theoretical” is
 132 here used for consistency.

133 An aluminium oxide ($\alpha\text{-Al}_2\text{O}_3$) was obtained from Interchim (pure 99.99%, theoretical size
 134 fraction 200 – 500 nm) and purified as described in Janot et al. (2012) ²².

135
 136

137 *Table 1: Theoretical molar fraction of the different NPs used in this study*

	<i>Au</i>	<i>Ag</i>	<i>Al</i>	<i>Co</i>	<i>Zn</i>	<i>Ni</i>	<i>Si</i>	<i>Ti</i>	<i>Fe</i>	<i>Mg</i>	<i>Ca</i>
80 nm Au-NPs	1.00	/	/	/	/	/	/	/	/	/	/
80 nm Ag-NPs	/	1.00	/	/	/	/	/	/	/	/	/
60 nm Ag@Au-NPs	0.13	0.87	/	/	/	/	/	/	/	/	/
80 nm Ag@Au-NPs	0.25	0.75	/	/	/	/	/	/	/	/	/
FeCoNi	/	/	/	0.17	/	0.17	/	/	0.67	/	/
FeCoZn	/	/	/	0.17	0.17	/	/	/	0.67	/	/
Alum. ox. – Al₂O₃	/	/	1.00	/	/	/	/	/	/	/	/
Kaolinite – KGa-2	/	/	0.46	/	/	/	0.51	0.02	0.01	/	/
Montm. – STx-1b	/	/	0.21	/	/	/	0.70	0.002	0.008	0.06	0.02

138 Sample preparation and analysis

139 The clay mineral samples and the aluminium oxide were each dispersed in 50 mL of ultrapure
 140 water at 100 mg L⁻¹ and vigorously shaken for 15 minutes. In order to remove bigger particles (*i.e.* > 1
 141 μm), suspensions were left to settle overnight, *ca.* 15 hours, and then the top 10 mL from each
 142 supernatant were sampled.

143 All suspensions (*i.e.* model ENPs and NNPs) were first individually analysed and then, two
 144 mixtures were prepared and analysed:

145 i/ a first mixture with the model ENPs (Ag-, Au-, Ag@Au-NPs and FeCoNi/Zn oxides)

146 ii/ a second mixture with the model NNPs (Al₂O₃, KGa-2 and STx-1b).

147 The interaction between ENPs and NNPs being beyond the scope of this article, no ENPs-NNPs
 148 mixture was prepared.

149 Prior to spICP-ToF-MS analysis, suspensions were sonicated for 60 seconds and freshly
 150 diluted using ultrapure water to avoid NP coincidences as necessary after reviewing initial
 151 measurements.

152 Single particle ICP-ToF-MS

153 The simultaneous multi-elemental spICP-MS analysis was performed on an ICP-ToF-MS
 154 (Vitesse, Nu Instruments). This instrument is able to record mass spectra from ²³Na to ²³⁸U without

155 interruption at 27.5 μ s per spectrum. By reducing the data using a high-power graphics processing unit
 156 during collection, uninterrupted acquisitions are possible for multiple minutes at these fast dwell-
 157 times. Therefore, 3 raw spectra were accumulated for each fully processed spectrum stored to the
 158 solid-state hard drive resulting in a total dwell-time of 83 μ s, still allowing the deconvolution of all NP
 159 peaks. Spectral processing included the subtraction of electronic noise and spectral baseline followed
 160 by an integration of specific mass regions for each isotope within the mass range measured. Table 2
 161 gives representative spICP-ToF-MS operating conditions and parameters. spICP-ToF-MS raw data
 162 were processed using the integrated software – *NuQuant* using the methodology described by Shaw &
 163 Donard (2016) ²³.

164 *Table 2: Instrument operating conditions and spICP-ToF-MS parameters*

<i>spICP-ToF-MS parameters</i>	
<i>Cones</i>	standard Ni cones
<i>Uptake rate</i> ^{\$}	0.4 L min ⁻¹
<i>RF power</i> ^{\$}	1100 W
<i>Ar Cool gas</i> ^{\$}	13.0 L min ⁻¹
<i>Ar auxiliary gas</i>	2.0 L min ⁻¹
<i>Ar nebulization gas</i> ^{\$}	1.0 L min ⁻¹
<i>Collision cell gas</i> ^{\$}	He at 0.016 L min ⁻¹ and H ₂ at 0.002 L min ⁻¹
<i>Acquired mass range</i>	23 amu – 245 amu
<i>Examined isotopes</i> [#]	²⁴ Mg, ²⁷ Al, ²⁸ Si, ⁴⁴ Ca, ⁴⁸ Ti, ⁵⁶ Fe, ⁵⁹ Co, ⁶⁰ Ni, ⁶⁶ Zn, ⁹⁰ Zr, ¹⁰⁷ Ag ¹³⁹ La, ¹⁴⁰ Ce and ¹⁹⁷ Au
<i>Dwell-time</i> [*]	83 μ s [*]
<i>Oxides</i> ^{\$}	0.2% for BaO/Ba

165 ^{\$} The instrument was tuned daily to ensure maximum sensitivity and stability, as well as low barium
 166 oxide formation. [#] Full spectra from ²³Na to ²³⁸U were acquired by the ICP-ToF-MS. However, to reduce the data
 167 processing time post-acquisition, the isotope set was reduced to key elements. This made processing possible in
 168 seconds rather than minutes when calculating the permutations of element coincidence in each particle. ^{*} In this
 169 case, the dwell-time represents the spectral-acquisition time.

170 Hierarchical agglomerative clustering

171 The post-processing strategy for graphical representation, advanced calculations and cluster
 172 analysis was programmed in Python 3.8 using calculated moles and masses exported from *NuQuant*.
 173 The machine learning library – *Scikit-learn* (<https://scikit-learn.org/stable/>) – and specifically its
 174 object – *AgglomerativeClustering* – was used to perform the hierarchical agglomerative clustering
 175 (HAC).

176 The hierarchical clustering consists of grouping clusters recursively either in an agglomerative
 177 or divisive way. The hierarchical agglomerative clustering (HAC) is here preferred. Each datapoint is

178 considered as an individual cluster and is then successively merged in clusters based on similarities²⁴.
 179 The usual output of the HAC is a 2-D tree-like structure. The so-called dendrogram illustrates the
 180 arrangement of the grouped clusters^{20, 24}. The number of clusters is determined by cutting this
 181 dendrogram at a desired level (*i.e.* distance threshold discussed below). To classify the NPs within
 182 each sample, single stage clustering using the Ward minimum variance method calculated with the
 183 Euclidean distance is performed here as the recursive merging strategy. It is defined as the smallest
 184 increase in the within-cluster sum of squares due to the merging of two clusters. The Euclidean
 185 distance used here is the molar fraction normalized as a distance metric.

187 Results & Discussion

188 Characterization of individual suspensions of NPs

189 **Gold and/or silver NPs.** In this section, 80 nm pure Au- and Ag-NPs as well as 60 nm and 80 nm bi-
 190 metallic Ag shelled Au-NPs were individually analyzed using the ICP-ToF-MS in single particle
 191 mode.

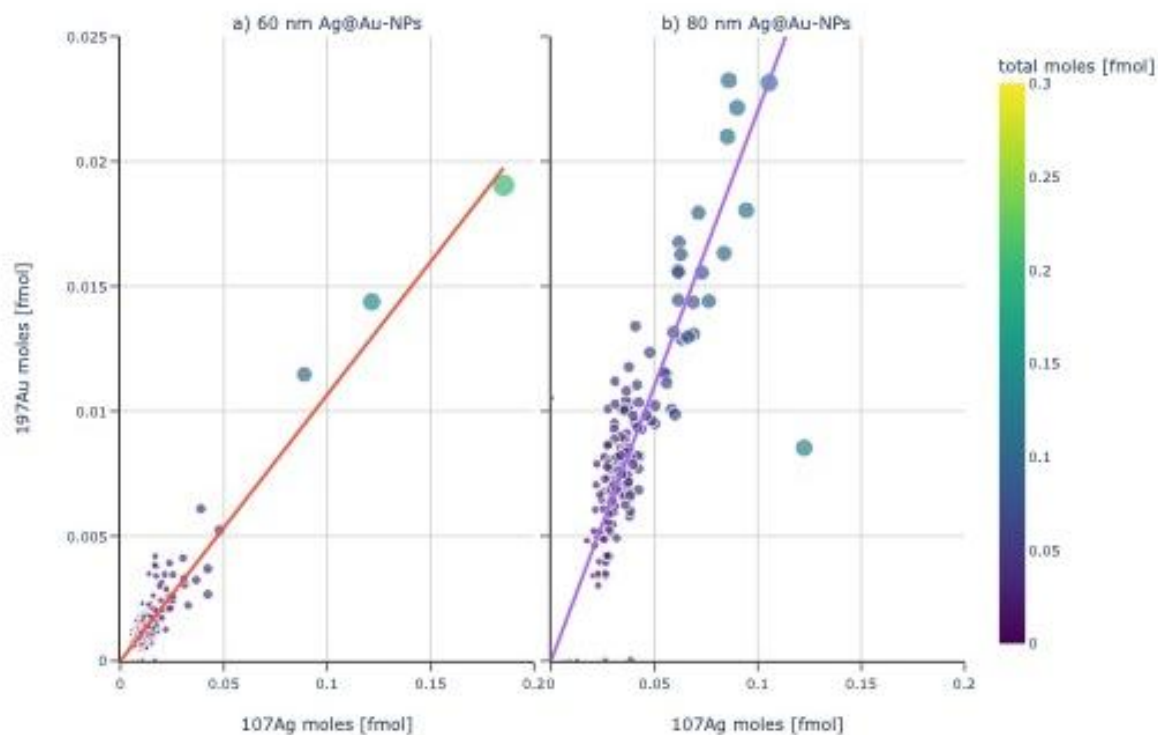
192 Figure 1 displays the number of moles of Au vs the number of moles of Ag (all in femtomoles and
 193 calculated using the sum of moles of the detected elements) for individual solutions.

194 Pure Au- and Ag-NPs (Figure 1 a and b) respectively give an average size of 76.6 ± 6.5 nm
 195 and 76.8 ± 13.0 nm (Figure 1 c and d) with a pure metallic fingerprint (molar ratio = 1). For 60 and 80
 196 nm bi-metallic Ag shelled Au-NPs, the average size per particle is respectively 63.2 ± 10.5 nm with a
 197 molar fraction of Ag = 0.90 ± 0.06 and Au = 0.10 ± 0.06 and; 93.7 ± 15.0 nm with a molar fraction of
 198 Ag = 0.83 ± 0.08 and Au = 0.17 ± 0.08 . Additionally, the calculated size of the Au-core is 29 ± 7 nm
 199 and 54 ± 10 nm for 60 and 80 nm for Ag@Au-NPs, respectively. Based on the average diameter of 60
 200 and 80 nm Ag@Au-NPs, the recalculated thickness of the Ag shell is respectively *ca.* 17 nm and *ca.*
 201 19 nm. These results show that calculated sizes as well as mole fraction of pure and bi-metallic NPs
 202 are in good agreement with the certified ones.

203 *Table 3: Experimental diameter and molar fractions of the different NPs for pure Au-, pure Ag- and bi-metallic Ag shelled*
 204 *Au-NPs. The theoretical molar fractions of 60 and 80 nm Ag@Au-NPs are 0.13/0.87 and 0.25/0.75, respectively*

	<i>Molar fraction</i>		<i>Nanoparticle diameter (nm)</i>
	<i>Au</i>	<i>Ag</i>	
80 nm Au-NPs	1.00	/	76.6 ± 6.5
80 nm Ag-NPs	/	1.00	76.8 ± 13.0
60 Ag@Au-NPs	0.10 ± 0.06	0.90 ± 0.06	63.2 ± 10.5
80 Ag@Au-NPs	0.17 ± 0.08	0.83 ± 0.08	93.7 ± 15.0

205



206
 207 *Figure 1: number of moles of Au vs the number of moles of Ag (all in femtomoles). Red and purple lines represent the best fit*
 208 *lines, for a) 60 nm and b) 80 nm Ag@Au-NPs respectively. The theoretical molar fractions of 60 and 80 nm Ag@Au-NPs are*
 209 *0.13/0.87 and 0.25/0.75, respectively. The colorbar gives the total amount of moles (in femtomoles) in individual datapoints*
 210 *while the size of each point is related to the calculated mass of each NP. Note that the molar fraction, the total amount of*
 211 *moles and the mass are calculated using the sum (mole or mass) of the elements detected within the NP*

212 **Nickel/Zinc Cobalt Iron oxide NPs.** Individual suspensions of tri-metallic Nickel, Cobalt, Iron and
 213 Zinc, Cobalt, Iron oxide NPs were analyzed by spICP-ToF-MS. Figure 2 displays the calculated molar
 214 fraction of the three metals in each suspension. In both tri-metallic NP solutions, 3 types of NPs are
 215 found with the following composition:

- 216 1/ pure Fe NPs,
- 217 2/ bi-metallic Fe-Co NPs
- 218 3/ tri-metallic Fe-Co-Ni/Zn NPs.

219 The average particle mass (sum of the mass of the detected elements) displayed in Table 4 shows that
 220 bi-metallic NPs have mass lower than tri-metallic NPs.

221 Considering first only tri-metallic NPs, calculated molar fractions (Table 4) are in good
 222 agreement with theoretical ones displayed in Table 1. Calculated sizes (assuming a spherical shape)
 223 are much larger compare to the theoretical values (*ca.* 98 ± 25 and 124 ± 30 nm, for Fe-Co-Ni and Fe-
 224 Co-Zn respectively, instead of 40 nm). These results suggest that only the larger fraction of the size
 225 distribution of the material or aggregates could be detected instead of the average size of individual tri-
 226 metallic NPs. It is confirmed in Figure SI-5 where the minimum size detectable of tri-metallic NPs has
 227 been computed assuming a spherical shape and a homogeneous theoretical composition such as
 228 $\text{Ni}_{0.5}\text{Co}_{0.5}\text{Fe}_2\text{O}_4$ and $\text{Zn}_{0.5}\text{Co}_{0.5}\text{Fe}_2\text{O}_4$. This leads to respective D_{min} of *ca.* 80 and 87 nm which are

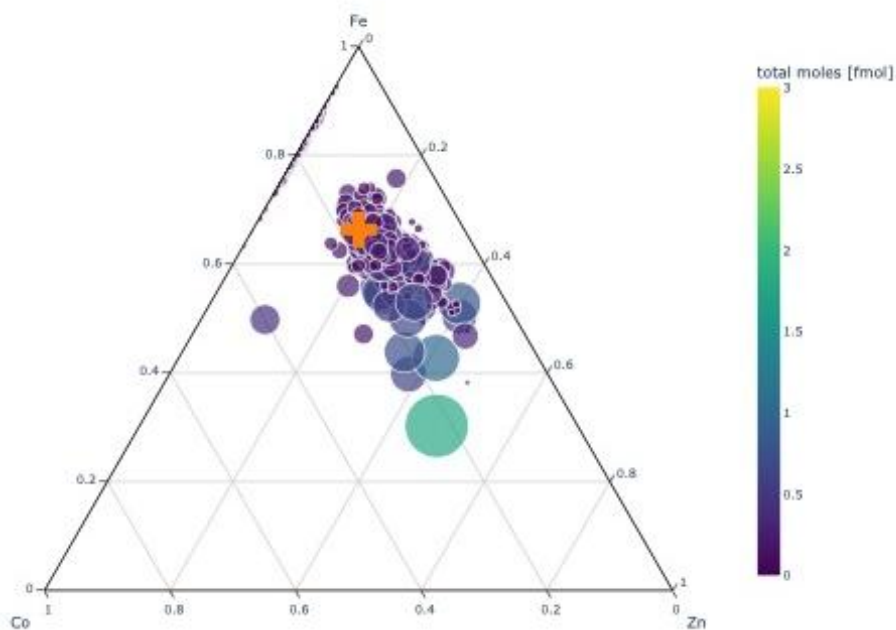
229 significantly above the theoretical size of 40 nm. Taking into account pure and bi-metallic NPs, two
 230 hypotheses can be made. Assuming a homogeneous composition of all NPs, higher M_{min} for Ni and
 231 Zn compare to Co displayed in Table SI-2 imply that if the NP mass decreases, the ability of the ICP-
 232 ToF-MS to detect Ni and Zn is first affected. This then exhibits NPs with a bi-metallic (Fe-Co)
 233 signature. For even smaller NPs, although Table SI-2 shows that $M_{min-Fe} > M_{min-Co}$, as Fe is the major
 234 constituent of the NP (omitting oxygen) this favors its detection which leads to the measurement of
 235 NPs with an apparently pure Fe composition.

236 A second assumption is that the elemental composition of these NPs is heterogeneous and that
 237 pure and bi-metallic NPs are indeed present within the suspension. The heterogeneity observed in
 238 Figure SI-1b first tends to confirm the dispersity of the tri-metallic NP composition as well as the
 239 presence of mono and bi-metallic NPs (Figure 2). In addition, as the size of the datapoint is related to
 240 the mass of the NPs in Figure 2, it clearly appears that there is no direct link between these masses and
 241 the presence of one or more elements. Therefore, this confirms that that mono-, bi- and tri-metallic
 242 NPs coexist in the solution.

243 *Table 4: Experimental mass and molar fractions of the different types of NPs coming from Fe-Co-Ni/Zn oxides. The*
 244 *theoretical molar fractions of Fe-Co-Ni/Zn oxides are 0.67/0.17/0.17*

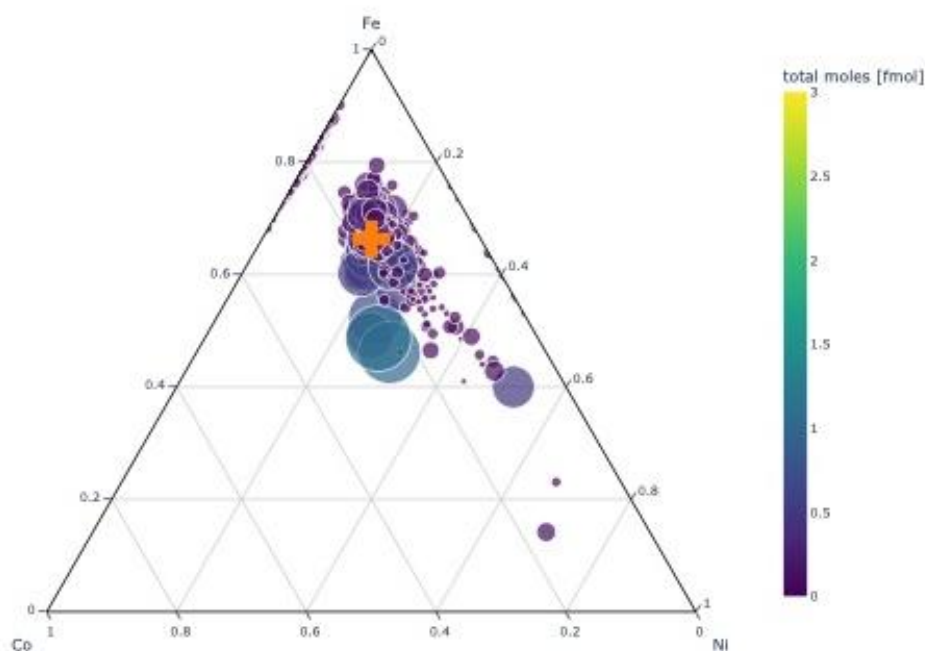
		<i>Molar fraction</i>			<i>Average particle</i>
		<i>Fe</i>	<i>Co</i>	<i>Ni/Zn</i>	<i>mass(fg)</i>
<i>Fe-Co-Ni</i>	<i>Fe</i>	1.00	/	/	0.5 ± 0.2
	<i>Fe-Co</i>	0.80 ± 0.03	0.20 ± 0.03	/	1.1 ± 0.6
	<i>Fe-Co-Ni</i>	0.66 ± 0.05	0.16 ± 0.03	0.18 ± 0.05	5.0 ± 5.2
<i>Fe-Co-Zn</i>	<i>Fe</i>	1.00	/	/	0.4 ± 0.2
	<i>Fe-Co</i>	0.82 ± 0.05	0.18 ± 0.05	/	1.4 ± 1.3
	<i>Fe-Co-Zn</i>	0.63 ± 0.04	0.14 ± 0.03	0.23 ± 0.05	9.5 ± 8.3

a)



245

b)

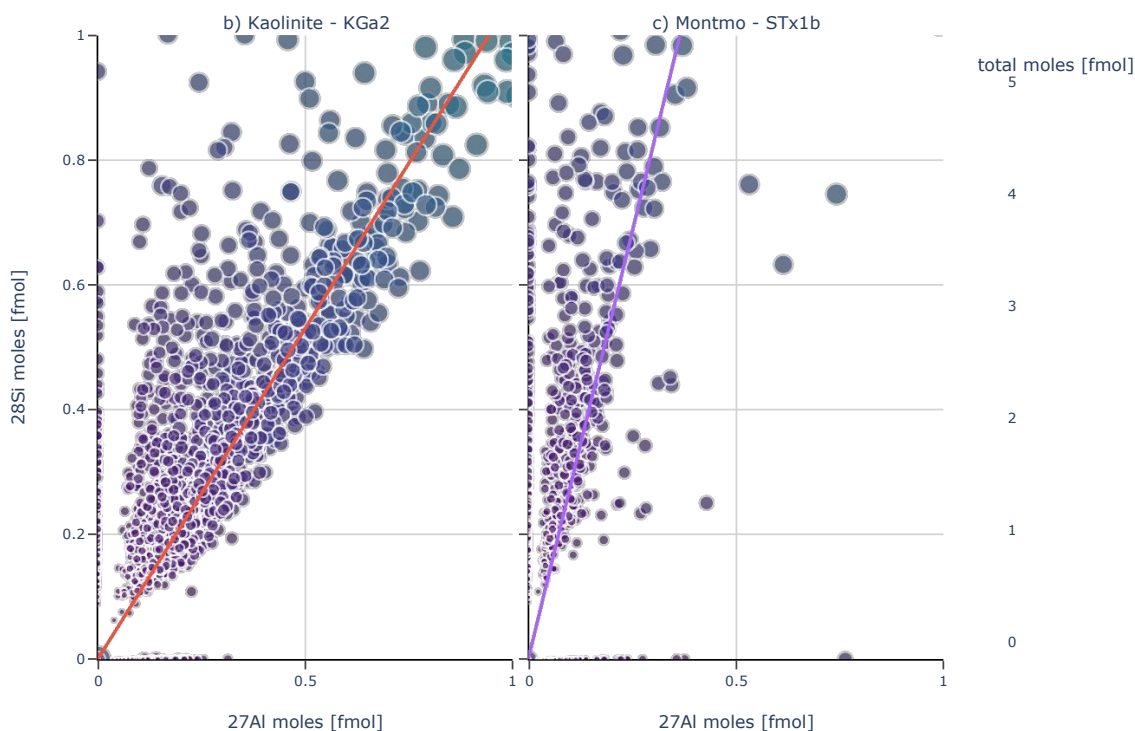


246

247 *Figure 2: Ternary diagrams of molar fractions of a) Fe-Co-Zn and b) Fe-Co-Ni. Orange cross represents the theoretical*
 248 *molar fraction (i.e. 0.67/0.17/0.17). The color bar gives the total amount of moles (in femtomoles) in individual datapoints*
 249 *while the size of each point is related to the calculated total mass of each NP. Note that the molar fraction, the total amount*
 250 *of moles and the mass are calculated using the sum (mole or mass) of the elements detected within the NP*

251 **Analogue of natural NPs.** In this section, the three model NNPs (i.e. a kaolinite, a montmorillonite as
 252 well as an aluminium oxide) are individually characterized using single-particle ICP-ToF-MS. Figure
 253 3 displays the number of moles of Si vs the number of moles of Al (i.e. the two major elements) for
 254 each sample. Fitted lines clearly appear to be different for all samples. For KGa-2 and STx1-b, lines of
 255 least squares fit are 1.06 and 2.73, respectively. These fits represent the experimental Si-Al molar
 256 ratio. Theoretical ones, calculated from the data in Table 1, are 1.11 and 3.33 for KGa-2 and STx1-b,

257 respectively. For the kaolinite, the experimental and theoretical molar ratio are in good agreement. For
258 the montmorillonite the experimental ratio slightly diverges from the theoretical one. The presence of
259 pure Al-NPs (datapoints displayed on the x-axis in Figure 3 c) decreases the correlation coefficient and
260 skews the line of fit but this latter is still very distinct from the one of kaolinite.
261



262

263 *Figure 3: (Zoom of Figure SI-3a for KGa-2 and STx-1b only). Number of moles of Si vs the number of moles of Al (all in*
264 *femtomoles). Red and purple lines represent the best fit lines, for KGa-2 and STx-1b, respectively. The theoretical Al-Si*
265 *molar fraction is 1.11 and 3.33 for KGa-2 and STx-1b, respectively. The color bar gives the total amount of moles (in*
266 *femtomoles) in individual datapoints while the size of each point is related to the calculated total mass of each NP. Note that*
267 *the molar fraction, the total amount of moles and the mass are calculated using the sum (mole or mass) of the elements*
268 *detected within the NP*

269 Identification of model ENPs in mixtures

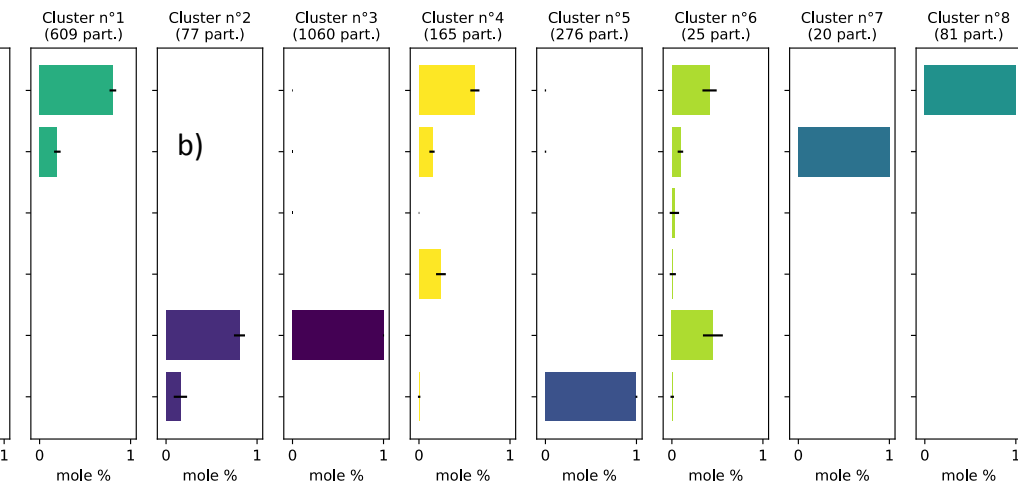
270 **Mixture of model ENPs.** The results displayed in the previous sections show that the characterization
271 of multi-metallic NPs is possible using an ICP-ToF-MS in single particle mode. Although sizes
272 provided for the tri-metallic NPs are much larger than expected, sizes of other NPs as well as all molar
273 fractions are individually, precisely determined. To go further, it is important to demonstrate that this
274 kind of analysis is precise and reliable for the identification of NPs in mixtures with a number of
275 monitored elements > 3 as in environmental samples. In order to mimic these kinds of samples, a
276 mixture of the model ENPs characterized above is analyzed by spICP-ToF-MS. Then, the hierarchical
277 agglomerative clustering (HAC) approach described above is used to recursively group similar events
278 (*i.e.* NP) with similar fingerprints (*i.e.* elemental molar fractions). The HAC, being an unsupervised

279 methodology, performs the clustering “blind” (*i.e.* without influence of a human being). However, as
280 mentioned above, the clustering must be stopped at some level by applying a threshold. Here, this
281 latter was visually determined after careful examination of the dendrogram and in order to obtain
282 minimum dispersion within each cluster. The optimized threshold distance obtain was $d_{\text{threshold}} = 3$.
283 Based on the summary displayed in Figure 4, the methodology looks precise and accurate. Indeed,
284 cluster characteristics are in agreement with the model ENPs described above. For instance,
285 considering silver and gold, the pure (cluster 3 and 5) and bi-metallic (cluster 2) populations are
286 displayed with the correct molar fractions. Equivalent spherical diameters also correspond to the ones
287 calculated when analyzed separately (Figure 4 c). For the tri-metallic NPs, pure Fe (cluster 8), bi-
288 metallic Fe-Co (cluster 1) as well as tri-metallic Fe-Co-Ni/Zn (cluster 0 and 4) NPs are still displayed
289 with molar fractions (Figure 4 a) and equivalent spherical diameters (Figure 4 c) in agreement with
290 information provided in the previous section. Two other clusters (cluster 6 and 7) are also displayed in
291 Figure 4 a. The mono-metallic composition (*i.e.* only Co is detected) as well as the very low mass
292 (Figure 4 b) of cluster 7 indicate that these NPs are probably pure Co-NPs dispersed during the
293 sonication and initially categorized as hot-spots in STEM-XEDS mapping (Figure SI-1b). Cluster 6
294 shows an unexpected multi-metallic composition with molar fraction of *ca.* Ag = 0.50, Fe = 0.45, Co =
295 0.04 and traces of Ni and Zn. The sample was initially diluted to reduce the probability of
296 coincidences. However, such NP coincidences as well as (hetero-)aggregation within the sample are
297 probable. Time-based graphic records displayed in the supporting information demonstrate that these
298 are easily identified through the continuous acquisition at tens of micro-second dwell-times. In the
299 first scenario (Figure SI-4a), the starting time of the peaks of Fe, Co, Zn and Ag is exactly the same.
300 Based on the NPs initially dispersed in the sample, this indicates a hetero-aggregation between a tri-
301 metallic NP and a pure Ag-NP. The Figure SI-4b shows a different scenario. Indeed, the starting point
302 of the peak of Ag is different than the one of Fe, Co and Ni indicating that an overlap is observed.
303 Thus, the way data are processed in *NuQuant* leads to an estimate that these pure Ag and tri-metallic
304 peaks are actually a single quadri-metallic peak. Although important to note that for future studies and
305 subsequent interpretations, a careful consideration of the raw data should be made to avoid
306 misinterpretation, the proportion of cluster 6 as well as cluster 7 is here negligible compared to the
307 others (*i.e.* 0.9% and 0.7%) (Figure 4 d).

308 This demonstrates the applicability of spICP-ToF-MS analysis coupled to HAC for model
309 ENPs identification. It especially shows that it is very efficient in differentiating populations even in
310 low proportions and also in highlighting (hetero-)aggregation. As described above, after examining the
311 dendrogram, an optimized distance threshold (*i.e.* $d_{\text{threshold}} = 3$) is used to end the clustering. The more
312 complex the dataset is, the more complicated the definition of the threshold becomes. For instance, by
313 increasing the distance threshold for this dataset, the total number of clusters decreases as some
314 clusters are merged together. The first populations to be merged are tri-metallic NPs both with Ni and
315 Zn giving one cluster with a quadri-metallic signature. Then by increasing the $d_{\text{threshold}}$ further, all

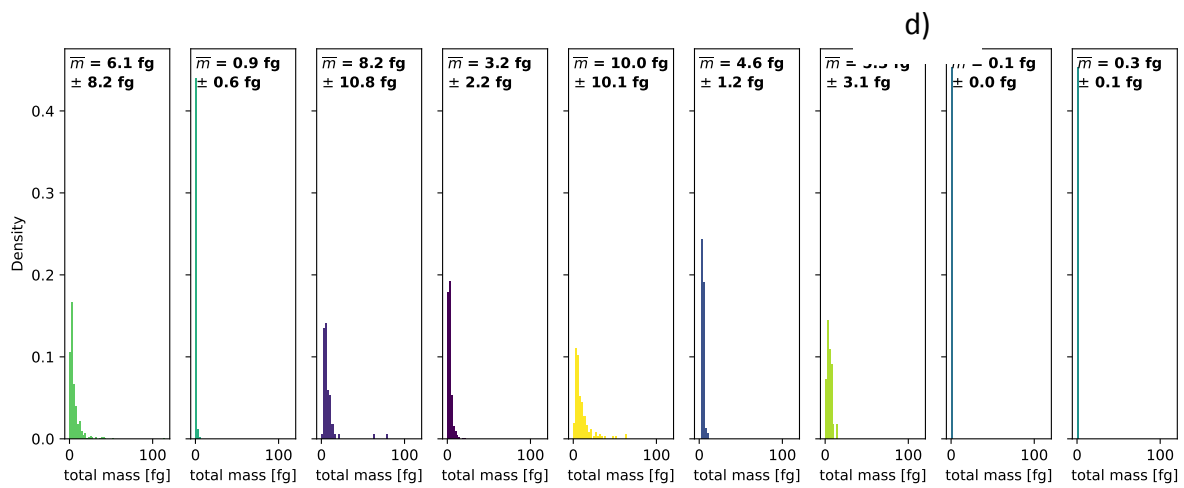
316 clusters containing either Fe, Co, Ni or Zn are merged together while pure Ag and Ag@Au-NPs are
317 also combined, leaving a third cluster with only Au-NPs. To find the appropriate/optimal distance
318 threshold in HAC (as for environmental samples), Krishnamoorthy et al. (2012)²⁵ propose an
319 Optimized Agglomerative Clustering (OAC) that reduces computational complexity and
320 misclassification errors with good accuracy using multilevel thresholds for finding the optimum
321 number of clusters. However, this optimized methodology is not yet implemented in Python but is a
322 promising line of development for future studies. In the next section, about the clustering of model
323 NNPs, in order to more objectively group the data, the distance threshold was fixed at the previously
324 optimized value found for the clustering of the model ENPs mixture *i.e.* $d_{\text{threshold}} = 3$.

a)

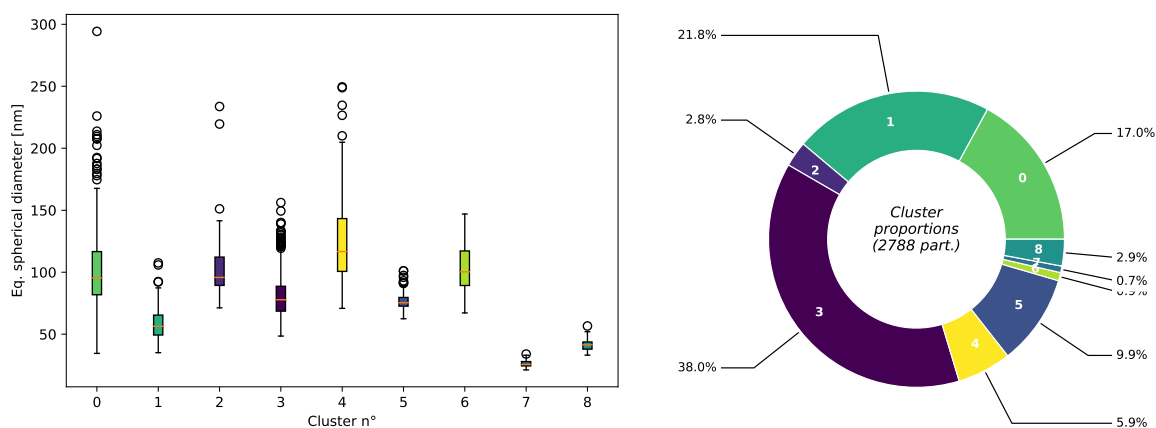


c)

325



326



327
 328
 329 *Figure 4: Summary of the hierarchical agglomerative clustering on spICP-ToF-MS data of the model ENP mixture analysis.*
 330 *a) average molar fractions in color and standard deviation in black, b) particle mass distributions calculated with the mass*
 331 *of all detected elements in the cluster, c) equivalent spherical diameters and d) proportions of individual clusters are*
displayed

332 **Mixture of model NNPs.** Previous results for model ENPs have shown that the here described
 333 methodology of coupling spICP-ToF-MS analysis with hierarchical agglomerative clustering is
 334 reliable for distinguishing them when mixed together as well as determining their individual
 335 fingerprints. Furthermore, spICP-ToF-MS has also been proved to be effective for the characterization
 336 of individual model NNPs. Thus, in the following section, model NNPs mixed together are analyzed
 337 using spICP-ToF-MS. Results are then processed using the HAC approach with the d_t previously
 338 optimized with the model ENPs. As for the mixture of model ENPs, our methodology preserves the
 339 molar fractions of each individual cluster/NP population (Figure 5). As expected, cluster 0 presents a
 340 (nearly-)pure-Al composition (*i.e.* Al molar fraction = 1) while clusters 1 and 4 are respectively
 341 composed of Si – Al – Fe – Ti and Si – Al – Mg – Fe – Ca – Ti. Looking at their molar fractions, these
 342 compositions are in agreement with the molar fractions of the kaolinite and the montmorillonite (Table
 343 1). Indeed, it appears that in cluster 1, silicon and aluminium respectively represent 0.50 ± 0.04 and
 344 0.49 ± 0.03 , followed by Ti and Fe with 0.007 ± 0.025 and 0.003 ± 0.005 clearly showing a KGa-2
 345 signature. Cluster 4 has silicon, aluminium and magnesium with respective molar fraction of $0.74 \pm$
 346 0.07 , 0.23 ± 0.07 and 0.02 ± 0.03 in agreement with the signature of STx-1b. Fe, Ca and Ti are less
 347 present in this cluster with molar fractions of 0.003 ± 0.004 , 0.002 ± 0.006 and 0.001 ± 0.003 ,
 348 respectively. Two additional (nearly-)pure NP clusters (*i.e.* Si (cluster 3) and Ti (cluster 5) with molar
 349 fraction *ca.* 0.99 ± 0.02) are also identified. As mentioned on the CMS website, quartz (SiO_2) was
 350 detected in STx-1b using infrared spectroscopy at $\lambda = 697 \text{ cm}^{-1}$. Titanium containing clusters are likely
 351 to be TiO_2 NNPs. These TiO_2 NNPs were previously observed by micro-Raman spectroscopy within clay
 352 mineral samples and particularly in kaolinite where Ti is as free-oxide at *ca.* 86%²⁶. Surprisingly, a
 353 last cluster (*i.e.* cluster 2) is displayed, still with Si – Al – Ti – Fe signature as for KGa-2 but with
 354 unexpected molar fractions ($0.66 \pm 0.06 - 0.33 \pm 0.07 - 0.009 \pm 0.021 - 0.004 \pm 0.005$, respectively).
 355 Note that this cluster is also present when analyzing a suspension of kaolinite only (Figure SI-3b). We
 356 therefore assume that this population is originally in the kaolinite and that this study is the first to

357 highlight its presence. Going a step further and combining this population with the one displayed in
 358 cluster 1, the Si – Al – Fe – Ti molar fraction of the kaolinite appears to be now $0.55 \pm 0.08 - 0.44 \pm$
 359 $0.06 - 0.003 \pm 0.004 - 0.007 \pm 0.016$, respectively.

360 However, this shows that coupling spICP-ToF-MS with HAC can distinguish model NNP populations
 361 with close signatures and accurately establishes their elemental fingerprint as well as correctly
 362 determines their mass distribution in a mixture (Figure 5).

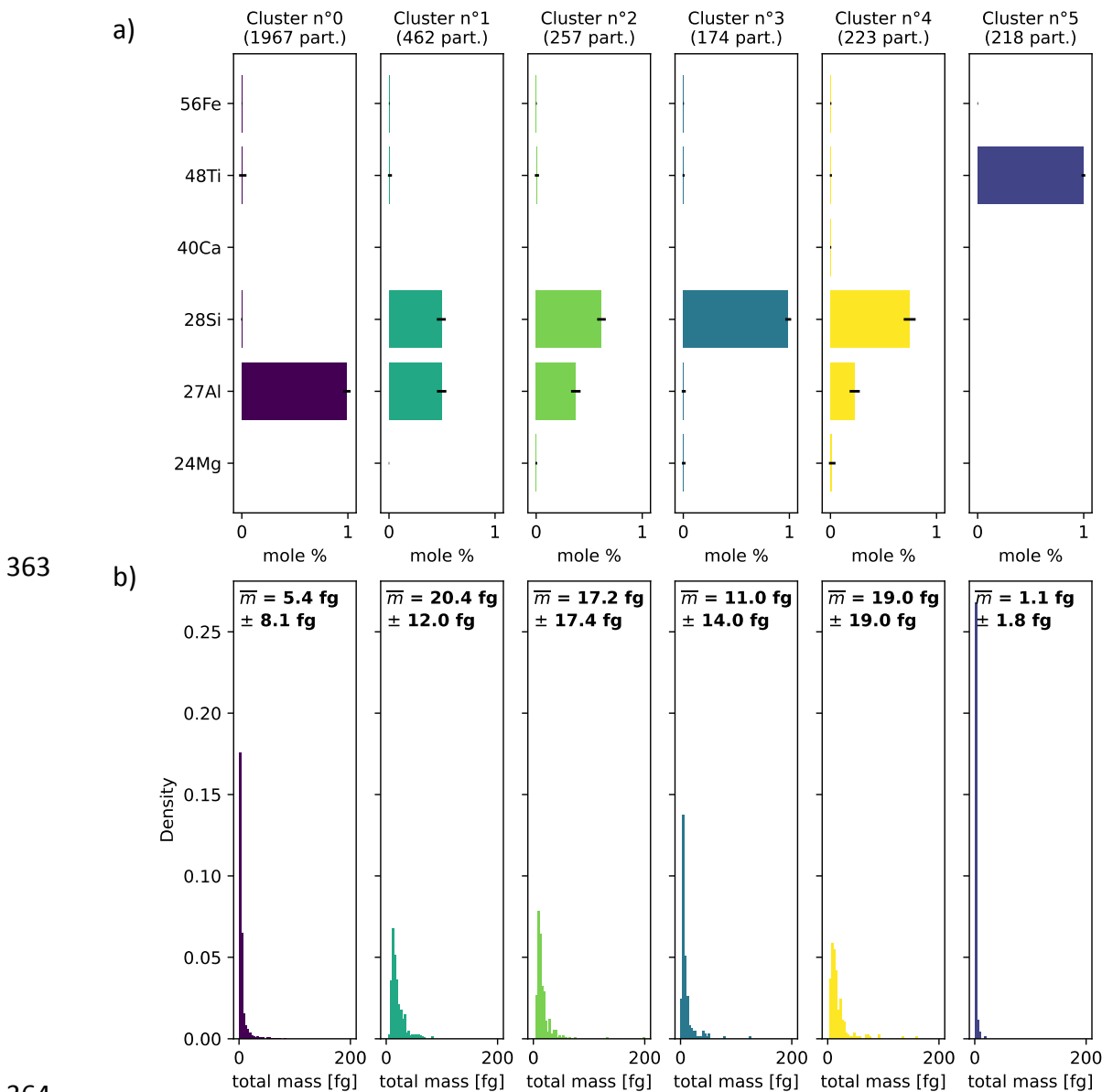


Figure 5: Summary of the hierarchical agglomerative clustering on spICP-ToF-MS data of the model NNP mixture analysis. a) Molar fractions and b) particle mass distributions of individual clusters are displayed

367 Conclusion

368 We have successfully analyzed model engineered and natural NPs using an ICP-ToF-MS in
 369 single particle mode as well as processed data of mixtures using an HAC approach. Using this

370 methodology, we first fully determined the elemental fingerprint of model ENPs alone and then mixed
371 together with good precision and accuracy. STEM analysis has confirmed the results qualitatively (*i.e.*
372 bi- or tri-metallic composition of the model NPs). We have subsequently provided elemental
373 composition and mass distribution information about a mixture of three types of model NNPs. The
374 presence of Ti- and Si-NPs also highlighted here is in agreement with infrared spectroscopy data
375 (CMS website) as well as μ -Raman spectroscopy data²⁶. For the first time in kaolinite, the presence of
376 a second population with a distinct Si-Al ratio was also demonstrated.

377 This work has been a starting point to better identify/classify NP populations (*i.e.*
378 anthropogenic *vs* natural NPs). However, in future studies, thorough evaluation of the data must be
379 performed in order to avoid misclassification of the NPs. Other factors such as low particle numbers as
380 well as low masses of NPs (such as cluster 6 and 7 in the mixture of model ENPs) must be
381 meticulously justified. Moreover, we have highlighted that the determined elemental composition is
382 affected by the sensitivity of the mass spectrometer. Indeed, as illustrated with the results of the tri-
383 metallic NPs, M_{min} of each element must be taken into account before assuming the origin of NPs.
384 Experiments on a variety of NP populations, must also be performed. Therefore, along with necessary
385 studies of natural environments, future work must also focus on supplying elemental signatures of
386 model engineered or natural NPs either alone or mixed together. By then comparing such studies with
387 unknown samples, the identification, origin and potential transformations of NPs should be possible.

388 From an analytical point of view, in order to increase reliability of the hierarchical clustering,
389 the automatic determination of the distance threshold should be improved as this is the starting point
390 of the grouping of NPs based on their similarities. Indeed, HAC miscomputation can obviously lead to
391 misinterpretation of spICP-ToF-MS data. Additionally, as demonstrated here, traces can be more
392 difficult to detect, limiting the understanding of the origin and the transformation of the NPs in the
393 media studied. Indeed, differentiating natural from engineered NPs is made through the detection of
394 elemental impurities within the NPs. Hence, manufacturers should continue to work on the
395 development of more efficient ion extraction and/or detection systems.

396

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441

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447 interpreting the TEM measurements.

448

449 **Figure captions**

- 450 • Figure 1: number of moles of Au vs the number of moles of Ag (all in femtomoles). Red and
451 purple lines represent the best fit lines, for a) 60 nm and b) 80 nm Ag@Au-NPs respectively.
452 The theoretical molar fractions of 60 and 80 nm Ag@Au-NPs are 0.13/0.87 and 0.25/0.75,
453 respectively. The colorbar gives the total amount of moles (in femtomoles) in individual
454 datapoints while the size of each point is related to the calculated mass of each NP. Note that
455 the molar fraction, the total amount of moles and the mass are calculated using the sum (mole
456 or mass) of the elements detected within the NP
- 457 • Figure 2: Ternary diagrams of molar fractions of a) Fe-Co-Zn and b) Fe-Co-Ni. Orange cross
458 represents the theoretical molar fraction (i.e. 0.67/0.17/0.17). The color bar gives the total
459 amount of moles (in femtomoles) in individual datapoints while the size of each point is
460 related to the calculated total mass of each NP. Note that the molar fraction, the total amount
461 of moles and the mass are calculated using the sum (mole or mass) of the elements detected
462 within the NP
- 463 • Figure 3: (Zoom of Figure SI-3a for KGa-2 and STx-1b only). Number of moles of Si vs the
464 number of moles of Al (all in femtomoles). Red and purple lines represent the best fit lines, for
465 KGa-2 and STx-1b, respectively. The theoretical Al-Si molar fraction is 1.11 and 3.33 for
466 KGa-2 and STx-1b, respectively. The color bar gives the total amount of moles (in
467 femtomoles) in individual datapoints while the size of each point is related to the calculated
468 total mass of each NP. Note that the molar fraction, the total amount of moles and the mass
469 are calculated using the sum (mole or mass) of the elements detected within the NP
- 470 • Figure 4: Summary of the hierarchical agglomerative clustering on spICP-ToF-MS data of the
471 model ENP mixture analysis. a) average molar fractions in color and standard deviation in
472 black, b) particle mass distributions calculated with the mass of all detected elements in the
473 cluster, c) equivalent spherical diameters and d) proportions of individual clusters are
474 displayed
- 475 • Figure 5: Summary of the hierarchical agglomerative clustering on spICP-ToF-MS data of the
476 model NNP mixture analysis. a) Molar fractions and b) particle mass distributions of
477 individual clusters are displayed

478 **Table captions**

- 479 • Table 1: Theoretical molar fraction of the different NPs used in this study
480 • Table 2: Instrument operating conditions and spICP-ToF-MS parameters

- 481 • Table 3: Experimental diameter and molar fractions of the different NPs for pure Au-, pure
482 Ag- and bi-metallic Ag shelled Au-NPs. The theoretical molar fractions of 60 and 80 nm
483 Ag@Au-NPs are 0.13/0.87 and 0.25/0.75, respectively
- 484 • Table 4: Experimental mass and molar fractions of the different types of NPs coming from Fe-
485 Co-Ni/Zn oxides. The theoretical molar fractions of Fe-Co-Ni/Zn oxides are 0.67/0.17/0.17