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1 Detection and characterization of biogenic selenium 2 nanoparticles in selenium-rich yeast by single particle ICPMS

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14 15 ABSTRACT

16 A method based on single particle inductively coupled plasma mass spectrometry (SP-ICPMS) was
17 developed for the analysis of commercial Se-rich yeasts, to confirm the occurrence of selenium
18 nanoparticles in these food supplements. A considerable reduction of the background levels was
19 achieved by combining data acquisition at microsecond dwell times and the use of H₂ reaction cell,
20 improving by a factor of 10 the current state-of-the-art methodology, and bringing size detection

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3 21 limits down to 18 nm for selenium nanoparticles. The presence of nanoparticulate selenium was
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5 22 unveiled by size-exclusion chromatography ICPMS, detecting a selenium peak at the exclusion
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7 23 volume of the column showing absorption at the wavelength corresponding to selenium
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9 24 nanoparticles. SP-ICPMS allowed to confirm the presence of Se-nanoparticles, as well as to
10
11 25 calculate the nanoparticle size distribution, owing to the information about the shape and elemental
12
13 26 composition of the nanoparticles obtained by transmission electron microscopy (TEM) and energy
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15 27 dispersive X-ray spectroscopy (EDS), respectively. These results reveal the significance of
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17 28 nanoparticles in the speciation of metals and metalloids in biological samples and the capability of
18
19 29 SP-ICPMS in combination with TEM-EDS to carry out these analyses.
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31 **1. Introduction**

32 Tailored metal/metalloid biogenic nanoparticles with specific physiochemical properties have been
33 shown to be highly toxic to several pathogenic bacteria and may offer an attractive alternative for
34 therapy of infections by antibiotic resistant bacteria.^{1,2} In particular, selenium nanoparticles (SeNPs)
35 synthesized by microorganisms, such as bacteria, fungi or yeast were demonstrated to possess
36 antibacterial, antiviral and antioxidant properties.^{1,2} The process of the Se⁰ nanoparticle formation is
37 based on the reduction of a toxic selenite (SeO₃²⁻) or selenate (SeO₄²⁻) to the less toxic (for the host
38 organism) elemental selenium through the intra- or extracellular formation of SeNPs with a typical
39 spherical shape and a diameter of 50-400 nm.^{3,4}

40 Yeast is not only recognised as a model system to study selenite or selenate metabolic
41 detoxification pathways,⁵ but it has also been the basis of an important biotechnological process of
42 their conversion to selenoamino acids, in particular to selenomethionine.⁶ Indeed, yeast

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3 43 (*Saccharomyces cerevisiae*) grown on selenite or selenate media, accumulates up to 3000 $\mu\text{g g}^{-1}$ of
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5 44 selenium, and has been used as a food and feed supplement,⁶ and at high doses (>200 $\mu\text{g Se/day}$) in
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7 45 prostate and colon cancer prevention treatments.⁷ The subsequent authorizations obtained by several
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9 46 companies for the commercialization of Se-rich yeast were preceded by the development of
10
11 47 analytical methods for the specific identification and quantification of the different chemical forms
12
13 48 of selenium present (speciation) of which the state-of-the art was reviewed.⁸

14
15 49 The currently available analytical methods allow the determination of selenomethionine
16
17 50 [with a relatively high confidence owing to the availability of a certified reference material (SELM-
18
19 51 1)],⁹ selenocysteine and a water soluble metabolome fraction.⁸ They also allow the determination of
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21 52 the residual (non-reacted) selenite or selenate, referred to as “inorganic selenium” of which the
22
23 53 presence below 2% is considered by the legislators as a proof of an “organic” character of Se-rich
24
25 54 yeast.¹⁰ Our experience over the past decade, through the analysis of several hundred samples from
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27 55 about 20 different suppliers, indicates that the selenium mass balance for the identified species
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29 56 rarely exceeds 90% which would suggest the presence of unaccounted forms of selenium.

30
31 57 To our best knowledge, Se° has never been quantified in yeast, although there were some
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33 58 attempts to its quantification in garlic¹¹ and in *Thunbergia alata*¹² using operationally defined or
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35 59 chemical conversion methods. Nanometer-sized deposits were reported in yeast cells grown in the
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37 60 presence of selenium by using X-ray radiation fluorescence spectroscopy¹³ without being
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39 61 characterized more deeply or quantified. We are putting forward here a hypothesis that a certain
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41 62 amount of selenium may be present in Se-rich yeast supplements as SeNPs and are proposing the
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43 63 development of an analytical method for its verification.

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64 To date SeNPs produced by microorganisms have been characterized by transmission
65 electron microscopy (TEM),^{3,4,14-19} X-Ray diffraction (XRD)²⁰ and atomic force microscopy
66 (AFM).^{19,20} For instance, TEM analyses provided evidence of the formation of electron-dense
67 granules in Se-treated microorganisms which were absent in the control ones.^{3,4} The presence of
68 selenium in these granules could be confirmed by energy dispersive X-ray spectroscopy (EDS)
69 while the absence of peaks from other elements indicated the presence of Se in the elemental state
70 rather than as a selenide.^{3,4} TEM was also applied for the characterization of SeNPs produced by
71 *Bacillus cereus*,¹⁴ the soil bacterium *Pseudomonas putida* KT2440,¹⁵ the filamentous bacterium
72 *Streptomyces sp.* ES2-5,¹⁶ the rhizobacterium *Azospirillum brasilense*,^{17,18} and a genetically
73 modified *Pichia pastoris* strain.¹⁹ In the latter case, results were confirmed by AFM. On the other
74 hand, SeNPs of an average size of 21 nm produced by bacterial isolate *Pseudomonas aeruginosa*
75 strain JS-11 were characterized by XRD.²⁰ The morphology and size of the nanoparticles were
76 further validated by AFM. Recently, an alternative to TEM measurements was proposed by using
77 the capabilities of asymmetrical flow field-flow fractionation (AF4) coupled on- and off-line with
78 different detectors, such as diode array (DAD), dynamic light scattering (DLS) or inductively
79 coupled plasma mass spectrometry (ICP-MS)^{21,22}. For example, biogenic SeNPs synthesized by
80 lactic acid bacteria (LAB) were characterized by AF4 coupled on-line with DLS and results in good
81 agreement with those obtained by TEM and nanoparticle tracking analysis (NTA) were obtained²².
82 A major drawback of these methods is their difficulty to detect, characterize and quantify SeNPs at
83 low concentrations ($\mu\text{g kg}^{-1}$).

84 This limitation can be overcome by single particle inductively coupled plasma mass
85 spectrometry (SP-ICPMS), which is one of the emerging techniques for the detection,

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3 86 characterization and quantification of nanoparticles.²³ The theoretical basis of SP-ICPMS was
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5 87 outlined by Degueldre *et al.*²⁴ and further developed by Laborda *et al.*²⁵ SP-ICPMS is able to
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7 88 discriminate (detect and quantify) dissolved versus particulate forms of the element in a sample, and
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9 89 to provide information about the element mass content per particle. Moreover, if additional
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11 90 information about their composition, shape and density is available, the size of the particles can be
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13 91 obtained, as well as their number and mass concentration.²⁶

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16 92 The feasibility of SP-ICPMS is compromised by the achievable size detection limits. For
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18 93 elemental selenium nanoparticles, a detection limit of 200 nm was estimated theoretically.²⁷ This is
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20 94 by far too high for the microorganisms related applications, although this size detection limit was
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22 95 calculated by using the low abundant (9.36%) ⁷⁶Se isotope, due to inherent problems for selenium
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24 96 determination by ICPMS because of spectral interferences. This problem can usually be overcome
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26 97 by using mathematical correction equations²⁸ or reaction/collision cells²⁹.

27
28 98 The objective of this work is the development of a method for the detection and size
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30 99 characterization of selenium nanoparticles by single particle ICPMS with the aim to reduce
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32 100 considerably the size-detection limits predicted up to now.²⁷ The method is going to be used to
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34 101 verify the occurrence of inorganic nanoparticulate selenium in Se-rich yeasts, confirming the
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36 102 hypothesis that this species must be included in the speciation schemes of this element in Se-rich
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38 103 yeasts.

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42 43 105 **2. Experimental**

44 45 106 **2.1. Standards, samples and reagents**

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3 107 Diluted suspensions of gold and selenium nanoparticles were prepared from commercially available
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5 108 materials. A reference gold nanoparticle suspension RM 8013 of 60-nm nominal diameter was
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7 109 obtained from NIST (NIST, Gaithersburg, MD). Suspensions of selenium nanoparticles of nominal
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9 110 diameters of 50 and 100 nm were purchased from Nanocs (Nanocs, New York, NY). Dilutions were
10
11 111 prepared in ultrapure water by accurately weighing (± 0.1 mg) aliquots of the stock suspensions
12
13 112 after 1 min sonication (Branson 2510, Branson, Danbury, CT; nominal power and frequency: 100
14
15 113 W, 42 kHz \pm 6%). After dilution and before each analysis, the suspensions were bath sonicated for
16
17 114 1 min (same power and frequency). Longer sonication times were not used to avoid excessive
18
19 115 heating of the suspensions. Aqueous selenium solutions were prepared from a standard stock
20
21 116 solution of 10000 mg L⁻¹ (Sigma-Aldrich, St. Louis, MO) by dilution in ultrapure water. Ultrapure
22
23 117 water (18.2 M Ω cm) was obtained from a Milli-Q system (Millipore, Guyancourt, France).
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25 118 Selenium-rich Brewer's yeast samples (obtained from a commercial provider), corresponding to a
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27 119 yeast strain *Saccharomyces cerevisiae*, grown in different concentrations of selenium were used.
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31 120 32 121 **2.2. SP-ICPMS analysis and data processing**

33 122 An Agilent 7900 Inductively Coupled Plasma Mass Spectrometer (ICPMS) (Agilent, Tokyo, Japan)
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35 123 was used throughout. The sample introduction system consisted of a concentric nebulizer and a
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37 124 quartz cyclonic spray chamber. Default instrumental and data acquisition parameters are listed in
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39 125 Table 1. Settling time during data acquisition was eliminated and the total acquisition time was 60 s
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41 126 in all experiments.
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45 127 Nebulization efficiency was calculated according to the particle frequency method
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47 128 developed by Pace *et al.*³⁰ The sample flow rate was calculated daily by measuring the mass of
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129 water taken up by the peristaltic pump for two minutes. This operation was repeated three times and
 130 the average value used for calculations. Under the experimental conditions used along this work, the
 131 nebulization efficiency at a sample flow rate of 0.35 mL min⁻¹ was 3.5 %.

132 Dwell times of 5 ms and 100 μs were studied and isotopes ⁸⁰Se and ⁷⁸Se monitored. Single
 133 Nanoparticle Application Module for ICPMS MassHunter software (Agilent), as well as in-house
 134 developed programs based on MatLab (MathWorks, Natick, MA) and Excel (Microsoft, Redmond,
 135 OR) spreadsheets were used for data processing. OriginPro 8 data analysis software (OriginLab
 136 Corporation, Northampton, MA) was also used.

137
 138 **Table 1** Default instrumental and data acquisition parameters for single particle ICPMS

Instrumental parameters	
RF Power	1550 W
Argon gas flow rate	
Plasma	15 L min ⁻¹
Auxiliary	0.9 L min ⁻¹
Nebulizer	1.10 L min ⁻¹
Reaction cell flow rate (H₂)	5.0 mL min ⁻¹
Sample uptake rate	0.35 mL min ⁻¹
Data acquisition parameters	
Dwell time	5 ms, 100 μs
Readings per replicate	12000, 600000
Settling time	-
Total acquisition time	60 s
Isotopes monitored	⁷⁸ Se, ⁸⁰ Se, ¹⁹⁷ Au

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140 2.3. Size Exclusion chromatography – ICPMS

141 A Superdex Peptide 10/300 GL column (GE Healthcare, Pittsburgh, PA) was coupled to an Agilent
142 7700x ICPMS (Agilent) instrument. Chromatographic separations were performed by using a model
143 1200 series HPLC pump (Agilent) as a delivery system. The exit of the column was connected in
144 series to an UV-visible detector (Agilent) and the ICPMS instrument.

146 2.4. Transmission electron microscopy

147 Samples were prepared on holey carbon films on mesh copper grids. A few microliters of each
148 sample were dropped on the grid and left to dry completely. Images were obtained using a FEI
149 TECNAI 12 (FEI, Hillsboro, OR) and recorded using an ORIUS SC1000 11MPx (GATAN,
150 Pleasanton, CA) CCD camera. The microscope is equipped with an energy-dispersive X-ray
151 analysis system for elemental analysis.

153 2.5. Procedures

154 **2.5.1. Acid digestion.** The content of total selenium in selenium nanoparticle suspensions
155 and Se-rich yeast samples was determined by ICPMS following acid digestion in a DigiPREP
156 digestion system (SCP Science, Quebec, Canada). 250 μL of H_2O_2 (VWR International, Fontenay-
157 sous-Bois, France) and 1 mL of conc. HNO_3 (Baker, Deventer, Netherlands) were added to 250 μL
158 of sample. The digestion was performed at 65 $^\circ\text{C}$ for 4 h. After digestion the volume was made up
159 to 50 mL so that the final HNO_3 concentration was 2% (v/v). Digestions were made in duplicate.
160 Blanks were run in parallel with the samples, as well as Certified Reference Material SELM-1

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3 161 (National Research Council of Canada) was analysed in order to validate the total selenium
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5 162 determination after acid digestion.
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7 163 **2.5.2. Enzymatic digestion.** The digestion/extraction procedure included four steps: (1) 200
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9 164 mg of a Se-rich yeast sample were suspended in 5 mL of water, bath sonicated for 1 h and
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11 165 centrifuged at 4500 x g for 10 min; (2) the pellet was resuspended with a solution of 5 mL of
12
13 166 driselase (Sigma Aldrich, Saint-Quentin Fallavier, France) 4% (*m/v*) in Tris (Sigma Aldrich) 30
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15 167 mM at pH 7.5, incubated at 25°C for 17 h and centrifuged at 4500 x g for 10 min; (3) the pellet was
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17 168 resuspended with a solution of 5 mL of protease (Sigma Aldrich) of 4 mg L⁻¹ in Tris 30 mM at pH
18
19 169 7.5, incubated at 37°C for 17 h and centrifuged at 4500 x g for 10 min; (4) finally, the pellet was
20
21 170 resuspended with a solution of 5 mL of sodium dodecyl sulphate (SDS, Sigma Aldrich) of 4%
22
23 171 (*m/v*), bath sonicated for 1 h and centrifuged at 4500 x g for 10 min. The supernatant was recovered
24
25 172 and kept at 4°C until analysis. One of the samples was subjected to an extra step: 1 mL of the SDS
26
27 173 extract was digested with a 1 mL protease solution of 4 mg L⁻¹ in Tris 30 mM at pH 7.5, incubated
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29 174 at 37°C for 17 h and centrifuged at 4500 x g for 10 min.
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37 176 **3. Results and discussion**

38 177 **3.1. Selection of instrumental parameters for the improvement of size detection limits**

39 178 In SP-ICPMS, the size detection limit is critically dependent on the detection efficiency (ions
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41 179 arriving to the detector with respect to the atoms in the plasma) and the background signal. Isobaric
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43 180 and matrix/plasma polyatomic interferences, as well as dissolved species of the element measured
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45 181 contribute to the continuous baseline in the time scans recorded in single particle mode. The most
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182 significant effect of a high baseline level is the loss of capability to identify particles with smaller
183 amounts of the element measured, and hence the increase in the size detection limits.³¹

184 Selenium has 6 naturally occurring stable isotopes with abundances from 0.9 to 49.6%
185 which are severely interfered mostly by Ar containing ions (Table S1). This problem has been
186 overcome in quadrupole ICPMS by selecting less-interfered isotopes (e.g., ⁸²Se) or by using
187 mathematical correction equations.²⁸ However, the use of reaction/collision cells²⁹ should allow the
188 use of the most abundant isotopes ⁸⁰Se and ⁷⁸Se,^{32,33} which are otherwise severely interfered by Ar
189 dimers. The fact that the size detection limit value of 200 nm was estimated by Lee *et al.*²⁷ using
190 relatively-low abundant ⁷⁶Se (9.36%) suggests a large margin for improvement of the size detection
191 limit of SeNPs if a high abundance isotope is selected and the polyatomic interferences removed.
192 Finally, a decrease of dwell times from milliseconds to microseconds would result in a proportional
193 reduction of the absolute baseline level³⁴ and hence of the related noise. The above considerations
194 were the basis of the method optimization strategy.

195 **3.1.1. Choice of the isotope: effect of the collision cell.** Our goal was to use one of the two
196 most abundant selenium isotopes, ⁷⁸Se and ⁸⁰Se, by reducing or eliminating the background
197 interferences. Apart from the contribution of residual Kr in the Ar gas supply, the main plasma
198 background contributions at masses 78 and 80 arise from Ar dimers. Indeed, the direct measurement
199 at m/z 80 is not possible, as the background exceeds 6.4×10^7 cps (time scans are shown in Fig. S1);
200 a considerable background (4.1×10^4 cps) is also observed for ⁷⁸Se. The use of collision/reaction
201 cells to reduce argon-based polyatomic interferences has been previously reported in literature (but
202 never for selenium in the single-particle mode), with the use of different gases such as methane³⁵⁻³⁷
203 or a He-H₂ mixture.³² For instance, the potentially interfering argon dimers at the selenium masses

74, 76, 78 and 80 were reduced by approximately five orders of magnitude by using methane as reactive cell gas.³⁵ In our case, the pressurization of the collision cell with H₂ (5.00 mL min⁻¹) led to a 5 x 10³-fold decrease in the background for ⁷⁸Se (down to 8 cps) and 7 x 10⁵-fold for ⁸⁰Se (down to 90 cps). Therefore, the use of the reaction cell leads to an important decrease of background signals in both cases, being more pronounced at mass 80. If sensitivities at mass 78 with and without reaction cell are compared, an improvement of more than twice is observed using H₂, which is explained through the isotopic abundance of the isotopes. Table 2 summarizes the background signals, the standard deviation of the associated noise, the signal-to-noise ratio and the sensitivity (slope of the calibration curve for selenium water solution) which allowed the calculation of the attainable concentration detection limits for the different selenium isotopes in the standard and collision/reaction cell modes.

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Table 2 Background signals, associated noise (expressed as standard deviation of background), signal-to-noise ratio, sensitivity and attainable concentration detection limits for the different selenium isotopes when measured with and without collision/reaction cell. Dwell time: 100 μs

Se	H ₂ cell	Background (cps)	Noise (cps)	Sensitivity (cps L μg ⁻¹)	S/N	LD (μg L ⁻¹)
80	No	63700000	2160000	O/R	---	---
78	No	41300	18800	7700	0.04	7.33
80	Yes	90	995	40600	40.8	0.074
78	Yes	8	300	17800	59.33	0.051

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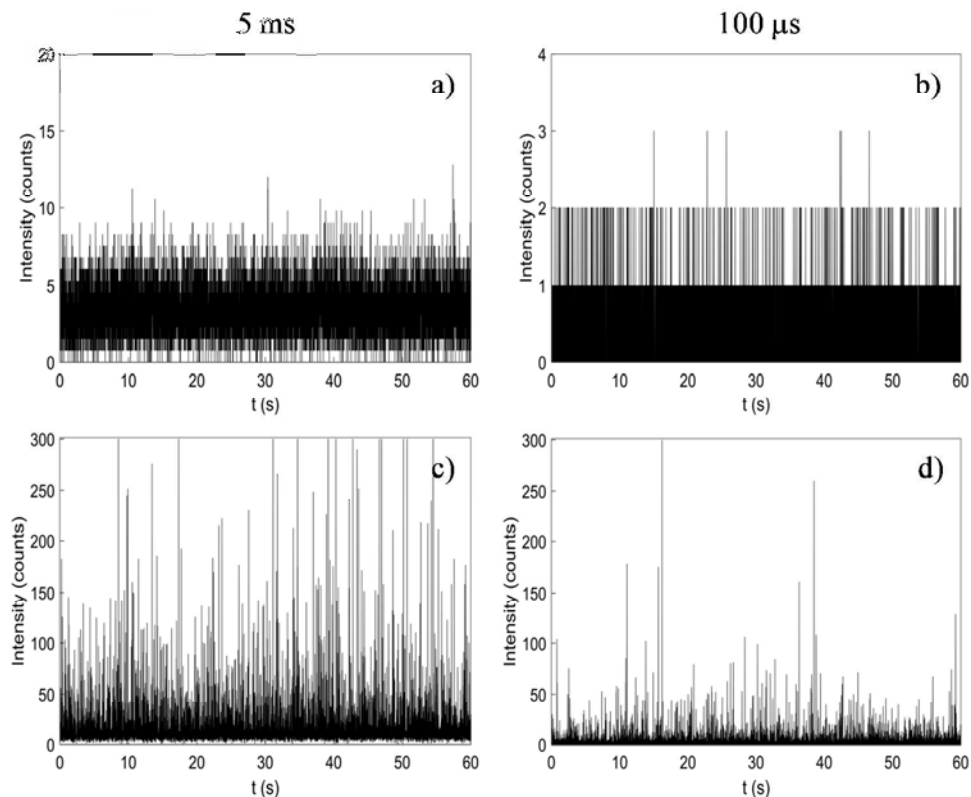
3.1.2. Dwell time. When a sufficiently diluted suspension of nanoparticles is introduced into the plasma, each particle produces an individual pack of ions which is detected as such. By

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3 222 using dwell times in the millisecond range (3-10 ms), events corresponding to the detection of
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5 223 single particles are detected as single pulses, whereas they are detected as transient signals when
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7 224 microsecond dwell times (<100-200 μ s) are selected. On the other hand, the dissolved species
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9 225 present together with the residual plasma background, produce a constant signal in the detector. The
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11 226 intensity of this signal, expressed in counts, decreases proportionally if dwell times are shortened³²
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13 227 whereas the corresponding noise diminishes according to the square root of the background (for
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15 228 signals below ca. 1000 counts, shot noise being the main contribution to the noise).²⁶

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17
18 229 Fig. 1 compares the time scans at dwell times of 5 ms and 100 μ s with reaction cell
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20 230 recorded for water and for a 50-nm selenium nanoparticle suspension with a nanoparticle number
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22 231 concentration of $\sim 1.85 \times 10^8 \text{ L}^{-1}$. When working in the milliseconds regime (Figs. 1a, c), an
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24 232 averaged baseline signal of 3.4 counts was measured. However, when the dwell time was shortened
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26 233 to 100 μ s (Figs. 1b, d) the intensity of the baseline was close to zero. Therefore, working in the
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28 234 microsecond range instead of the millisecond range, makes it possible to reduce the contribution of
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30 235 the background and thus to improve the size detection limits.

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238 **Fig. 1** ^{80}Se time scans of (a-b) ultrapure water, (c-d) 50-nm nanoparticle suspension of $1.85 \times 10^8 \text{ L}^{-1}$. Dwell times: 5 ms, 100 μs .

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241 3.2. Size detection limits

242 The intensity corresponding to the dissolved species or/and the background (μ_B) affects directly the
 243 attainable size detection limit (LOD_{size}) through its standard deviation (σ_B). Applying a 3σ
 244 criterion²⁵ for spherical, solid, and pure nanoparticles, and estimating σ_B as the square root of the
 245 background counts plus one³¹, the LOD_{size} is given by:

246

$$LOD_{size} = \left(\frac{18 \sigma_B}{\pi \rho X_{NP} K_{ICPMS} K_M} \right)^{\frac{1}{3}} = \left(\frac{18 \sqrt{\mu_B + 1}}{\pi \rho X_{NP} K_{ICPMS} K_M} \right)^{\frac{1}{3}} \quad (1)$$

247

248 where ρ is the density of the nanoparticles, X_{NP} the mass fraction of the element in the nanoparticle,
249 K_{ICPMS} the detection efficiency (ratio of the number of ions detected versus the number of atoms
250 introduced into the ICP), and $K_M (=AN_{Av}/M_M)$ includes the contribution from the element measured
251 (A, atomic abundance of the isotope considered; N_{Av} , Avogadro number; M_M , the atomic mass).

252 Apart from the influence of dissolved/background on LOD_{size} , Equation 1 includes the
253 detection efficiency, which depends on the particular instrument. On the other hand, the relationship
254 between the signal R (ions counted per time unit) and the mass concentration C^M of a solution of an
255 analyte nebulized into an ICPMS can be expressed as:

256

$$R = K_{intr} K_{ICPMS} K_M C^M \quad (2)$$

258

259 where $K_{intr} (= \eta_{neb} Q_{sam})$ represents the contribution from the sample introduction system,
260 through the nebulization efficiency (η_{neb}) and the sample uptake rate (Q_{sam}), whose values are
261 detailed in section 2. *Experimental*. By analysing a dissolved selenium standard and knowing the
262 value of K_{intr} , the term “ $K_{ICPMS} K_M$ ” can be deduced from Equation 2.

263 Table 3 summarizes the LOD_{size} calculated for different selenium isotopes and dwell times
264 in water. By monitoring the most abundant isotope ^{80}Se , working with H_2 as reaction gas and with a
265 dwell time of 100 μs , a size detection limit of 18 nm could be achieved, considering: $\mu_B = 0.0092$
266 counts; $\rho = 4.79 \text{ g cm}^{-3}$; $X_M = 1$; $\eta_{neb} = 0.035$; $Q_{sam} = 0.35 \text{ mL min}^{-1}$; $A = 0.4961$; $N_{Av} = 6.022 \times$

267 10^{23} ; $M_M = 78.96 \text{ g mol}^{-1}$; $R/C^M = 40600 \text{ cps L } \mu\text{g}^{-1}$. Taking the above into account, monitoring ^{80}Se
 268 and working in collision/reaction cell mode was concluded to be the best choice for the detection of
 269 SeNPs by SP-ICPMS.

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271 **Table 3** Size detection limits for selenium nanoparticles in water, nm

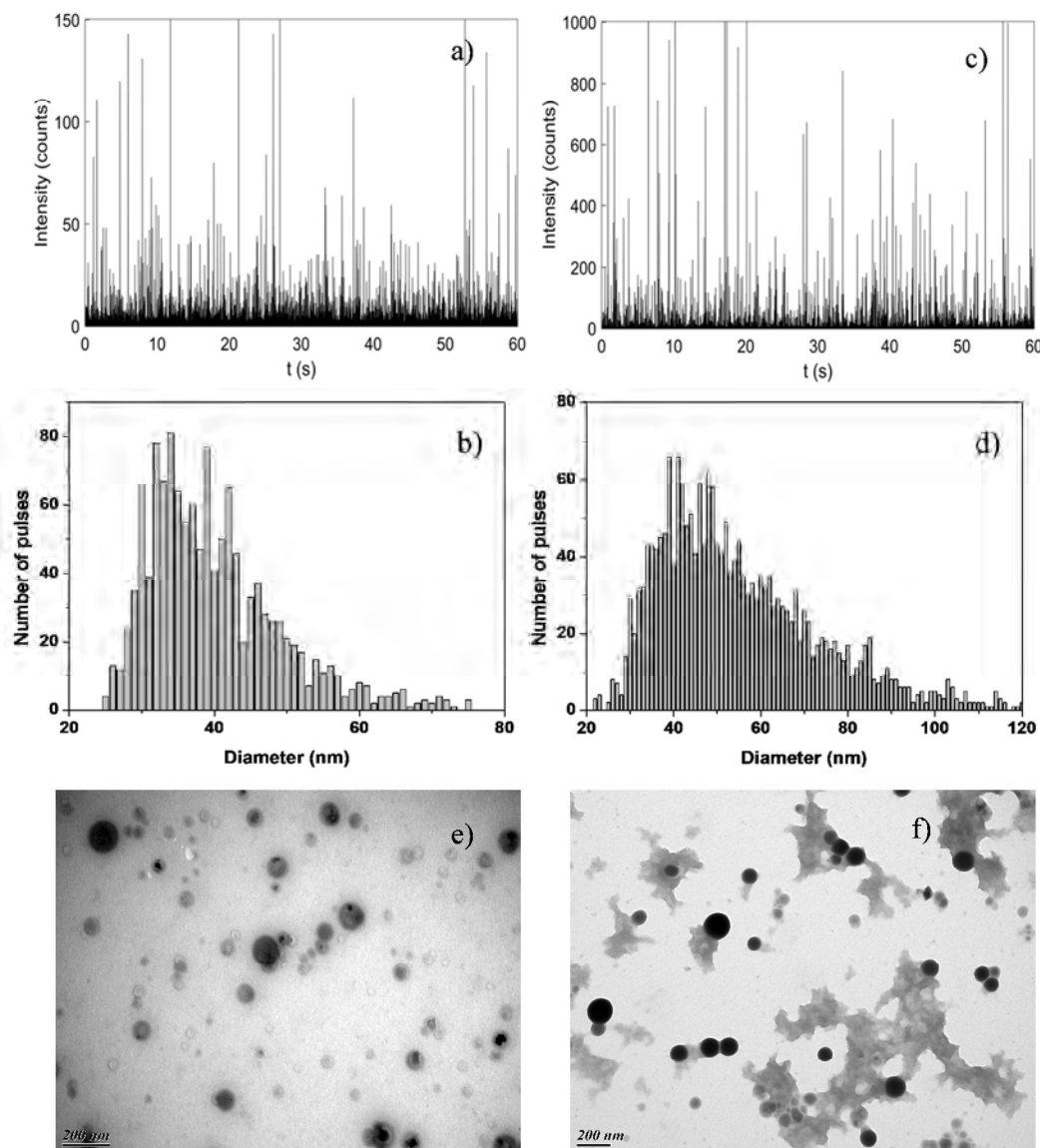
Dwell time	H ₂ cell	⁷⁸ Se, LOD _{size}	⁸⁰ Se, LOD _{size}
5 ms	No	83	-
	Yes	25	24
100 μs	No	43	-
	Yes	24	18

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273 **3.3. Analysis of commercial suspensions of selenium nanoparticles**

274 Two different commercial suspensions of SeNPs with nominal diameters of 50 and 100 nm were
 275 analyzed by the developed method. Fig. 2 shows the time scans and the corresponding number size
 276 distribution obtained for both commercial suspensions.

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279 **Fig. 2** ^{80}Se time scans of a) 50-nm Se nanoparticle suspension; c) 100-nm Se. Number size
280 distribution of b) 50-nm Se nanoparticle suspension; d) 100-nm Se. Dwell time: 100 μs .
281 Transmission electron microscope image of e) 50-nm Se nanoparticle suspension; f) 100-nm Se.
282 Scale bar: 200 nm.

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3 284 The developed method allowed the detection of SeNPs as small as 20 nm in diameter. The
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5 285 distributions showed an average diameter of 40.2 ± 0.4 nm for the nominal 50-nm particles and of
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7 286 57.1 ± 0.1 nm for the nominal 100-nm particles. TEM images for both suspensions are presented in
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9 287 Fig. 2e and 2f, showing spherical particles with no significant aggregation/agglomeration and some
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11 288 polydispersity. The corresponding size distributions have been included in Fig. S2, showing that the
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13 289 average sizes are in agreement with the nominal values (60 and 101 nm, respectively).

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15 290 The disagreement between TEM and SP-ICPMS results can be explained by a different
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17 291 response of the ICPMS towards the dissolved and the nanoparticulate selenium forms. To prove this
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19 292 hypothesis, the total content of selenium in the commercial suspensions of SeNPs was determined
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21 293 both by direct analysis of the diluted suspensions, and after their acid digestion. The calibration was
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23 294 achieved with aqueous standards of selenium in water and 2% HNO₃ respectively, since a
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25 295 dependence of the medium on the selenium sensitivity was observed. In order to verify the
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27 296 completeness of the digestion procedure, the corresponding digested solutions were also measured
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29 297 in single particle mode. No nanoparticle signals were observed, confirming that all the selenium
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31 298 present was in its dissolved form or as particles below 18 nm. The concentrations determined in
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33 299 water for the 50-nm and 100-nm SeNPs suspensions were 81.0 ± 3.4 % and 66.1 ± 7.6 % with
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35 300 regard to the concentration determined after acid digestion, respectively (Table 4). These results
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37 301 show that ICPMS sensitivity is dependent on the physicochemical form of selenium and on the size
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39 302 of the nanoparticles. If nebulization efficiency is considered equal for dissolved and particulated
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41 303 forms of selenium, the differences arises from the detection efficiency, most probably the less
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43 304 efficient atomization or ionization of selenium nanoparticles.

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306 **Table 4** Determination of selenium concentration in commercial suspensions of SeNPs in mg L⁻¹
307 (mean ± standard deviation)

Sample	50-nm	100-nm
Acid digestion	148.6 ± 5.5	92.1 ± 1.20
Suspensions in water	120.3 ± 2.2	60.85 ± 6.93

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310 The different behaviour observed for the dissolved and nanoparticulate forms of selenium
311 implies that the use of dissolved selenium standards for calculations of the selenium mass per
312 particle or the size of a selenium particle will produce a negative bias and hence SeNPs with similar
313 selenium content or size of the targets should be used as standards.

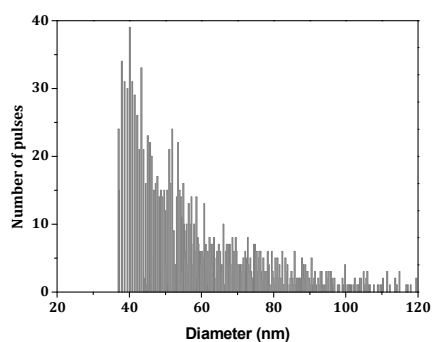
315 **3.4. Detection and characterization of biogenic selenium nanoparticles in Se-rich yeast** 316 **samples**

317 The developed method was applied for the detection and characterization of putative selenium
318 nanoparticles present in selenium enriched yeast.

319 **3.4.1. Enzymatic digestion of the yeast matrix.** Yeast samples were submitted to an
320 enzymatic digestion prior to their injection onto a size exclusion column for the separation of the
321 selenium-binding species as explained in section 2. *Experimental*. The effect of the digestion
322 procedure on the stability of SeNPs (dissolution or agglomeration) was also checked. For this, a Se-
323 free yeast sample was spiked with 100-nm SeNPs, submitted to the enzymatic digestion and
324 analyzed by SP-ICPMS. The size distribution obtained is shown in Fig. 3. In comparison with the
325 size distribution obtained for the original suspension (Fig. 2d), the size range was in good

326 agreement, proving that no agglomeration process occurred. However, obtaining the full
327 distribution was hampered by the presence of a relatively high background level, suggesting the
328 presence of dissolved selenium due to the partial oxidation of the nanoparticles.

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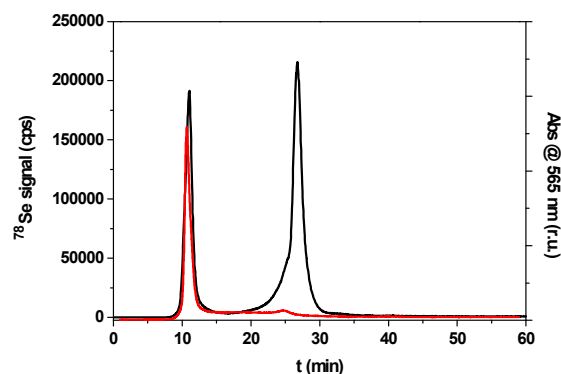
330 **Fig. 3** Number size distribution of 100-nm SeNPs suspensions after the enzymatic procedure.

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332 **3.4.2. Detection of selenium nanoparticles in yeast.** The chromatograms obtained for a
333 Se-rich yeast sample by size exclusion chromatography with Visible and ICPMS detection are
334 shown in Fig. 4. Low molecular weight species were eluted as a single peak at 28 minutes, whereas
335 another selenium containing peak was observed at the exclusion volume of the column (10 min).
336 Only the peak at the exclusion volume also showed absorption at 565 nm, a wavelength associated
337 to SeNPs,^{20,38} what suggests the presence of naturally occurring selenium nanoparticles in the yeast
338 sample.

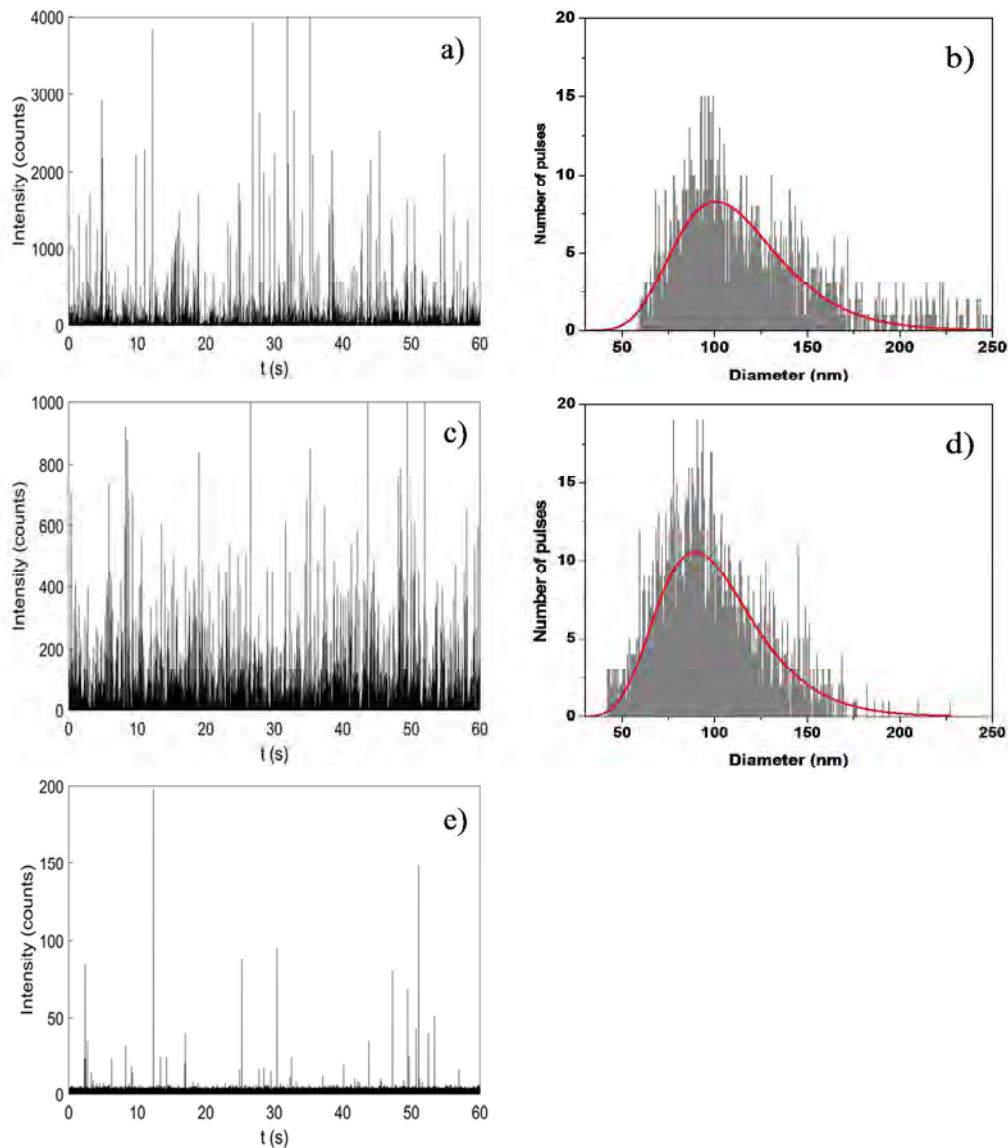
339 Furthermore, the sample of yeast after the digestion procedure (Sample A) and the collected
340 fraction corresponding to the exclusion volume in the chromatogram (Sample A post column) were
341 analysed by SP-ICPMS under the previously optimized conditions (monitoring ⁸⁰Se, with H₂

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3 342 reaction cell, dwell time: 100 μ s). The time scans obtained showed a significant number of signals
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5 343 above the background related to the presence of selenium-bearing nanoparticles in both cases (Figs.
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7 344 5a, c). These time scans were transformed into signals distributions (Fig. S3). A different Se-rich
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9 345 yeast sample (Sample B) was submitted to the same procedure and analysed by SP-ICPMS. In this
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11 346 case, only a few signals above the background were observed (Fig. 5e), meaning the presence of a
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13 347 small amount of selenium-bearing nanoparticles. This difference may be explained by the different
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15 348 total concentration values in the original samples. The original yeast of sample A contained 3000
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17 349 mg kg⁻¹ while the total selenium concentration in the original yeast of sample B was 2000 mg kg⁻¹.
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19 350 On the other hand, and in order to evaluate the particle detection capabilities of the method in real
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21 351 samples, the size detection limits of the different samples were calculated by using the background
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23 352 signal obtained in the time scans, obtaining values of 23, 20 and 19 nm for Sample A, Sample A
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25 353 post column and Sample B, respectively.
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356 **Fig. 4** Chromatograms of a selenium enriched yeast sample after the digestion procedure, obtained
357 by ⁷⁸Se signal (black signal) and Vis signal recorded at 565 nm (red line). The first peak
358 corresponds to the void volume of the column.

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361 **Fig. 5** ^{80}Se time scans and number size distributions obtained by SP-ICPMS corresponding to a-b)

362 Sample A; c-d) Sample A post column; and time scan corresponding to e) Sample B.

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3 364 **3.4.3. Size distribution of selenium nanoparticles.** SP-ICPMS provides information about
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5 365 the mass of element per nanoparticle, which means that the conversion into size involves knowing
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7 366 the actual composition, density and shape of the nanoparticles. Additional techniques like
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9 367 transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDS) were
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11 368 used to learn the shape and the elemental composition of the nanoparticles present in sample A.
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13 369 TEM images and EDS spectra obtained confirmed the presence of spherical nanoparticles whose
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15 370 composition was associated to elemental selenium (Fig. S4). Note that due to the low concentration
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17 371 of SeNPs in the sample, the number of particles detected in the images was too low to obtain a
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19 372 representative histogram, although a diameter around 100 nm could be measured from single
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21 373 images.

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23 374 Once the composition and the shape of the nanoparticles were determined, the time scans
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25 375 were transformed into size distributions (Figs. 5b, d), using the density of bulk elemental selenium
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27 376 (4.79 g cm^{-3}). Due to the different behaviour in the ICPMS with respect to the size and the
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29 377 physicochemical forms of selenium, as previously discussed, and to the size of nanoparticles
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31 378 observed by TEM ($\sim 100 \text{ nm}$), a sensitivity correction factor of 66 % was applied on the mass of
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33 379 selenium per nanoparticle, calculated by using aqueous standards of selenium in water. The size
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35 380 histogram obtained for Sample A showed a broad distribution of selenium nanoparticles, from 60 to
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37 381 200 nm (Fig. 5b). The size distribution was fitted into a log normal distribution and the median
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39 382 diameter was calculated, obtaining an average median diameter ($n=5$) of $108 \pm 4 \text{ nm}$ (average \pm
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41 383 standard deviation). On the other hand, a similar size distribution was obtained for the fraction
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43 384 collected at the exclusion volume of the column (Sample A post column, Fig. 5d), with an average
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45 385 median diameter of $97 \pm 3 \text{ nm}$ (average \pm standard deviation). These results are in good agreement
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386 with the data from TEM, where nanoparticles around 100 nm were observed and confirmed that a
387 process of biosynthesis of selenium nanoparticles occurred in selenium enriched yeast.

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389 **4. Conclusions**

390 An analytical method based on SP-ICPMS was developed for the detection and characterization of
391 SeNPs. The carefully optimization of parameters, including the monitored isotope, the choice of the
392 microsecond dwell time regime and the use of collision/reaction cell, allowed the reduction of the
393 background signal for using the most sensitive isotope of selenium. Under the optimal conditions, a
394 size detection limit of 18 nm could be obtained, which represents a gain of a factor of 10 in terms of
395 the prediction made elsewhere and the first ever single particle-ICP MS method for selenium
396 nanoparticle analysis. The method demonstrated the presence of SeNPs with sizes from 40 to 200
397 nm in Se-rich yeast and is able to provide information about the presence and size distributions of
398 nanoparticles at actual concentrations.

399

400 **Conflicts of interest**

401 There are no conflicts to declare.

402

403 **Supplementary Information**

404 Electronic supplementary information (ESI) available: Information about isotopic abundance and
405 spectral interferences for Se isotopes, time scans of ultrapure water, size distributions of SeNPs
406 suspensions, signal distributions of samples, TEM image and EDS spectrum.

407

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