Towards a method for detecting the potential genotoxicity of nanomaterials

Final Milestone report

Evaluation of the determination of Ti in tissues

April, 2013

This document arises from the NANOGENOTOX Joint Action which has received funding from the European Union, in the framework of the Health Programme under Grant Agreement n°2009 21.
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NANOGENOTOX **Grant Agreement n° 2009 21 01**

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Grant Agreement n° 2009 21 01

WP 7 : Toxicokinetics and tissue distribution of MNs for specification of organs at risk for genotoxicity testing (toxicokinetics)

Milestone report

Work Package leader RIVM

Wim H De Jong

National Institute for Public Health and the Environment

Antonie van Leeuwenhoeklaan 9, 3721 MA, Bilthoven, The Netherlands

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Workflow	
Author(s) Wim H De Jong (RIVM) Petra Krystek (Philips Innovation Services; former: MiPlaza) Yacine Nia, Sandrine Millour, Laurent Noël, Thierry Guérin (ANSES) Jutta Tentschert (BfR) Benedicte Trouillier, C. Denize, F. Ait-Ben-Ahmad, A. Papin (INERIS)	**Reviewer(s)** WP Leader: Wim H De Jong (RIVM) Coordinator: Nathalie Thieriet, Florence Etoré (ANSES)

Document status: Final version 8.
Creation date: 23/04/2013

Confidentiality level of the deliverable		
PU	Public	PU
CO	Confidential, only for members of the consortium (including the Commission Services)	

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1. Introduction

This report presents the results of an evaluation within the NANOGENOTOX Joint Action Plan for the determination of tissue titanium levels after treatment of animals with the nanoform of TiO_2. At the start of the NANOGENOTOX project the nanoparticles in the tissues could not be identified individually for determination of the tissue level, therefore the presence of total titanium was chosen as representative for the evaluation of the presence and distribution of the TiO_2 nanoparticles. After digestion of the tissues the Ti content was determined by inductively coupled plasma mass spectrometry (ICP-MS).

A major criterion for any toxicokinetic study is the possibility to detect the chemical under investigation. So, the study has to be performed with a dose sufficiently high to be able to detect the chemical under investigation. On the other hand the dose should not induce overt toxicity as this may have an effect on the absorption, distribution, metabolism and excretion (ADME) of the chemical. In order to determine a non toxic TiO_2 nanomaterial dose an acute toxicity study was performed.

For the performance of a toxicokinetic study of the tissue distribution of TiO_2 nanoparticles a method was developed for the evaluation of Ti in tissues by high resolution HR-ICP-MS by Philips Innovation Services (former MiPlaza Materials Analysis)/RIVM. In three additional Institutes (ANSES, BfR and INERIS) the same samples were evaluated for their Ti content by ICP-MS equipment available without and with different cell modes and settings.

2. Materials and Methods

Materials and Samples.

A TiO_2 nanomaterial solution was prepared according to the protocol developed in WP4 of the Nanogenotox project (Jensen et al. 2012). A 2.56 mg/ml stock dispersion was prepared by prewetting the amount of nanomaterial powder needed in 0.5 vol% ethanol (≥ 96% purity) followed by dispersion in 0.05 wt% rat serum albumin (RSA)-ultra pure water during 16 minutes of probe sonication on ice (Branson Sonifier S-450D, Branson Ultrasonics Corp., Danbury, CT, USA, equipped with a disruptor horn Model number: 101-147-037).

Tissue samples were obtained from rats after intravenous administration of the maximum TiO_2 dose possible with the NANOGENOTOX dispersion protocol (see Deliverable 3 WP4 The generic NANOGENOTOX dispersion protocol, Jensen et al. 2012). The stock solution was diluted for 10% using 10x concentrated phosphate buffer resulting in a final solution for administration to the animals of 2.3 mg/ml. So, the dose administered was 2.3 mg per animal, ranging from 8.6 to 10.3 mg/kg depending on the actual weight (range 224 – 266 g) of the animal.

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For the evaluation of the Ti determination organ samples were collected from animals in the acute toxicity study for determining a non toxic dose. Six-week-old male Wistar rats (HsdCpb:WU) were treated with a single intravenous dose of NM-100 and NM-102, 2.3 mg per animal in the acute toxicity study. No signs of toxicity were observed and animals were autopsied at day 14 after exposure. Several organs (liver, lung, spleen, heart, kidney, brain and muscle) were collected as a pilot study for determination of the Ti content ICP-MS. Organs were collected from 4 animals, 2 animals (#2 and #3) treated with NM-100 and two animals (#12 and #13) treated with NM-102. The organs were homogenized by manual cutting and stirring, and divided in several fractions and stored at -20°C at RIVM. Samples were sent to Philips Innovation Services (series A), ANSES (series B), BfR (series D), and INERIS (series E) for further evaluation. In addition, liver tissue samples of three vehicle treated animals were evaluated as well.

In the various assays six different pure MNs were studied in the Nanogenotox project (Table 1). These TiO_2 MNs were characterized by WP4. Table 1 shows the complete TiO_2 MNs selected material characteristics obtained in the Joint Action.

Table 1 Summary of samples and average data on key analytical results (given by WP4)

	Powder					
Sample	**Phase**	**Average XRD crystallite size**	**Average TEM particle size**	**Average BET & SAXS SSA£**	**TGA mass-loss**	**Main elemental impurities**
NM-100	anatase	56.7 - >100 nm	110 nm	9 m^2/g	nd	K,P
NM-101	anatase	7 nm	6 nm	316 m^2/g	8 wt%€	Al,Na,P,S,Zr
NM-102	anatase	21 nm	22 nm	78 m^2/g	nd	S
NM-103	rutile	23 nm	25 nm	51 m^2/g	2 wt%€	Al,Si,Na,S
NM-104	rutile	23 nm	25 nm	56 m^2/g	2 wt%€	Al,Si,Ca,Na,S
NM-105	anatase rutile	23 nm 60 nm	24 nm 15 nm	46 m^2/g	nd	nd

€ ascribed to organic coating/functionalisation; £ The average of one BET[1] and one SAXS[2] determination. $Note that DLS[3] size of CNT is an apparent number; * Not sizeable, nd: not detected.

3. Philips Innovation Services

3.1 Methods

[1] Brünauer, Emmett and Teller gas adsorption
[2] Small Angle X-ray Scattering
[3] Dynamic Light Scattering

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Method as developed at Philips Innnovation Services (former MiPlaza Materials Analysis), Eindhoven, The Netherlands.

Sample pre-treatment by digestion

A literature study was performed with the main focus on the digestion of titanium (dioxide), which is hardly soluble. In addition related literature was searched regarding the application of HR-ICP-MS especially for the use of evaluating various biological matrices. The two most relevant publications were:

1. Lomer M et al., Determination of titanium dioxide in Foods using inductively coupled plasma optical emission spectroscopy. Analyst, 2000, 125, 2339-2343.

2. Sarmiento-Gonzalez et al., Titanium levels in the organs and blood of rats with a titanium implant in the absence of wear, as determined by double-focusing ICP-MS. Analytical and Bioanalytical Chemisty, 2009, 393, 335-343.

After initial evaluation of several procedures, the following digestion procedure was used:

The sample material (approximately 0.5 g tissue) was weighed into 15 mL polypropylene tubes. Afterwards, 0.5 mL ultrapure water, 1 mL nitric acid conc (HNO_3) and 0.75 ml hydrofluoric acid conc. (HF) were added, and the tubes were placed on a block heater (type: Stuart SBH200D; supplied by Omnilabo, Breda, The Netherlands). The mixture was slowly heated to a final temperature of 90^{o}C and the mixture was left for two days at this temperature. Afterwards, ultrapure water was added to a total volume of 15 mL.

HR-ICP-MS Measurement

The measurements were performed with a HR-ICP-MS (High-Resolution Inductively Coupled Plasma Mass Spectrometer, ELEMENT XR, Thermo Fisher, Germany), using a set-up with on-line addition of an internal standard. The used sample introduction system and instrumental operating conditions of HR-ICP-MS (ELEMENT XR) set-up is summarized in Table 2.

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The element Ti consists in total of five naturally abundant isotopes. Nevertheless, three of these Ti isotopes cannot be used for quantification by HR-ICPMS due isobaric and polyatomic interferences. The abundances as well as the not resolvable, isobaric interferences are:

- ^{46}Ti with an abundance of 8.0% is interfered by calcium (^{46}Ca);
- ^{48}Ti with an abundance of 73.8% is interfered by calcium (^{48}Ca);
- ^{50}Ti with an abundance of 5.4% is interfered by chromium (^{50}Cr) and vanadium (^{50}V).

The MR mode was chosen for ^{47}Ti and ^{49}Ti as the following possible polyatomic interferences which can be resolved by using this MR mode, do not interfere the measurement of Ti:

- ^{47}Ti with an abundance of 7.3%: $^{31}P^{16}O$, $^{11}B^{36}Ar$, $^{7}Li^{40}Ar$, $^{15}N^{16}O_2$, $^{14}N^{16}O_2{}^{1}H$, $^{12}C^{18}O^{16}O^{1}H$ (see also Figure 1)
- ^{49}Ti with an abundance of 5.5%: $^{33}S^{16}O$, $^{13}C^{36}Ar$, $^{31}P^{18}O$, $^{9}Be^{40}Ar$, $^{14}N^{18}O^{16}O^{1}H$

The solutions were measured against an external calibration with internal standard correction. Two different internal standards (IS) were tested: ^{69}Ga, respectively ^{115}In and both internal standards (Ga, respectively In) can be used for this application. The methodical parameters are given in Table 3. For evaluation of the method used and quality control the general principles as described in the Eurachem Guide “The fitness for purpose of analytical methods (1998)” were applied.

Table 2. Sample introduction system and instrumental operating conditions of HR-ICP-MS (ELEMENT XR)

Sample introduction system	
Nebulizer	MicroMist Nebulizer 0.2 mL/min; from Glass Expansion, Australia
Operation mode	Pumped with on-line mixing of internal standard, 1:1
Spray chamber	Twister Spray Chamber with Helix; from Glass Expansion, Australia
Cones	Nickel, type H
Instrumental operating conditions	
RF power	1225 W
Cool gas flow	16 L/min Ar
Auxilary gas flow	0.9 L/min Ar
Sample gas flow	0.99 L/min Ar

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Table 3. Method and measuring parameters using HR-ICP-MS (ELEMENT XR)

Medium resolution mode (R=4000)	
Isotope (internal standard)	Used for quantification: ^{47}Ti (^{115}In) Used for control: ^{49}Ti (^{115}In)
Acquisition/'mass' window	125%
Search window	50%
Integration window	60%
Samples per peak	20
General parameters	
Acquisition mode	E-scan

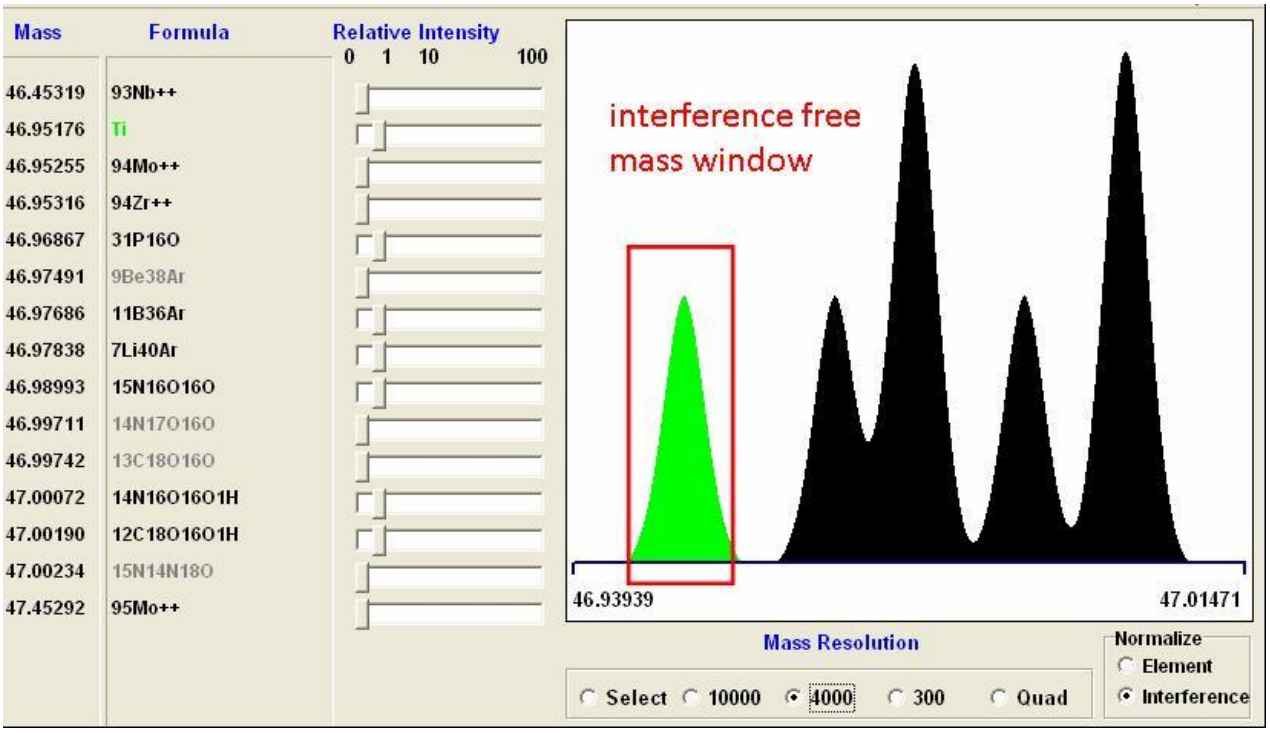

Figure 1. Simulation of ^{47}Ti determination in medium resolution (MR) mode of HR-ICP-MS as used for Ti determination.

Quality control experiments

1. Determination of presence of Ti in polypropylene tubes used and related correction of the raw data.
2. Analysis of independent duplicate samples from the original TiO_2 dispersions.
3. Spike experiments

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For developing the method initial tests were performed using spiking of the nanomaterials with tissues of nontreated control animals. The materials used for these initial studies were TiO_2 NM-103 and NM-105 provided as powders.

4. Control materials

As there is no certified reference material available for TiO_2 nanoparticles in a biological matrix, two batches of a commercially available reference blood sample with the total concentration of Ti (given as additional analytical values) were used; Seronorm Trace Elements Whole Blood; Sero AS Norway (L-2 LOT 1003129 with values of 18 ± 5 µg Ti/L and 19.5 ± 3.1 µg Ti/L, L-3 LOT 1112691 with a value of 12.8 ± 0.4 µg Ti/L).

3.2 Results

3.2.1 Sample treatment/digestion

At Philips Innovation Services (former MiPlaza Materials Analysis), Eindhoven, The Netherlands, three different digestion procedures were tested with the aim of achieving the most complete digestion of the sample material and regarding to an efficient handling in the case of applying the same procedure later on for large series of biological samples from the biodistribution studies.
For these tests, the original NPs (TiO_2 nanomaterials NM-103 or NM-105) as well as different organ and blood samples (amount: approx. 0.4 g organ resp. 0.5 ml blood spiked with several mg original NPs) were pretreated and analysed according to the same procedure. NM-103 is a rutile hydrophobic nanomaterial. NM-105 is a rutile-anatase mix of titanium dioxide.

1. Digestion with sulfuric acid in polypropylene tubes (based on Lomer et al., 2000):
Description:
The sample material (NM-103 or NM-105) was weighed into polypropylene tubes. After the addition of diluted sulfuric acid, the tubes were placed on a heating block system. The mixture was slowly heated to a final temperature of 65°C and the mixture was left overnight at this temperature.
Observation:
With the applied procedure, it was not possible to achieve a complete digestion of the original TiO_2 nanomaterial. The biological material was only partly digested and also formed a lot of black residue; no follow-up.

2. Digestion with sulfuric acid in glass tubes (based on Lomer et al. 2000):
Description:
The sample material (NM-103 or NM-105) was weighed into glass tubes. After the addition of sulfuric acid, the tubes were placed on a heating block system. The mixture was slowly

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heated to a final temperature of 200°C and the mixture was left overnight at this temperature.

Observation:

With the applied procedure, it was not possible to achieve a complete digestion of the original TiO_2 nanomaterial. The biological material was only partly digested and also formed a lot of black residue; no follow-up.

3. Digestion with a mixture of nitric acid and hydrofluoric acid in polypropylene tubes (based on Sarmiento-Gonzalez et al. 2009):

Description:

The sample material was weighed into 15 mL polypropylene tubes. Afterwards, 0.5 mL ultrapure water, 1 mL nitric acid conc. and 0.75 mL hydrofluoric acid conc. were added, the tubes were placed on a block heater (type: Stuart SBH200D; supplied by Omnilabo, Breda, The Netherlands). The mixture was slowly heated to a final temperature of 90°C and the mixture was left for two days at this temperature. Afterwards, ultrapure water was added to a total volume of 15 mL.

Observation:

With the applied procedure, it was possible to achieve a good digestion of the original TiO_2 NPs as well as of the biological materials. Prior to the measurement by HR-ICP-MS, the solutions were diluted. This procedure was used in the experiments by Philips Innovation Services for the determination of Ti content in tissue samples as follows (section 2 Materials and Methods).

3.2.2 Quality control experiments

1. Determination of presence of Ti in polypropylene tubes

Due to the fact that Ti can be also abundant in polypropylene tubes and in the used chemicals (acids and water), it is of great relevance to determine the blank concentration of Ti and to correct the raw analytical data for this concentration of Ti. The blank concentration of Ti was determined for 15 ml polypropylene tubes from three suppliers and these results are given in Table 4.

Table 4. Blank concentration of Ti concentrations from 15 mL polypropylene tubes and the used chemical

Supplier	Concentration (Ti) µg/L
Sarstedt	1.6 ± 0.1 (n=3)
Corning	1.6 ± 0.1 (n=3)
Greiner	2.3 ± 0.3 (n=3)

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During this study, in all series tubes from Sarstedt (Sarstedt BV, Etten-Leur, The Netherlands) were used and regularly controlled. The average of (n=41) is (1.4 ± 0.4) µg/L Ti. Within each series, the related blank concentration was used for correction.

2. and 3. Analysis of TiO_2 dispersions and spiked biological samples

For these experiments two elements (Ga and In) were tested as internal standards. The results obtained with the original TiO_2 samples are presented in Table 5. For the NM-103 a slightly lower amount was measured compared with the amount weighed. A difference was noted between NM-103 and NM-105 TiO_2 that might have been due to the hydrophobic nature of the NM-103 nanomaterial. Results of various biological samples spiked with TiO_2 nanomaterials are presented in Table 6. The recovery in the table is presented as percentage of the amount measured versus the amount added to the tissue samples. Regarding the recovery similar results were obtained for NM-103 and NM-105 as for the determination in the originally prepared TiO_2 dispersion. The measurement of NM-105 resulted in a level of Ti well above 90% and up to 100% of the Ti added, while for NM-103 the results were in the range of 85% to 90% of the amount originally added.

Table 5. Analysis of Ti present in original samples

Sample	Composition	Measured (%) IS (Ga)	Measured (%) IS (In)
NM-103	TiO_2 rutile, hydrophobic	85 ± 2	85 ± 3
NM-105	TiO_2 rutile+anatase	94 ± 5	94 ± 6

Results are presented as average of the results for ^{47}Ti and ^{49}Ti using either Ga or In as internal standard (IS). Data are presented as the amount of Ti measured as percentage of the Ti weighed and analyzed by HR-ICP-MS.

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Table 6. Analysis of various biological matrices spiked with TiO_2 nanoparticles expressed as the recovery of the spiked amount of Ti.

Samples	Recovery (%) IS (Ga) measured	Recovery (%) IS (In) measured
	Spiked with NM-103 TiO_2	
Testis	90	89
Testis	82	83
Heart	87	86
Blood	88	89
Blood	88	89
Average (n=5)	87 ± 3	87 ± 3
	Spiked with NM-105	
Testis	106	105
Testis	98	99
Heart	114	114
Blood	144	144
Blood	99	99
Average (n=5)	112 ± 19	112 ± 19

Results presented as average of the results for ^{47}Ti and ^{49}Ti. Data are presented as the amount of Ti measured as percentage of the amount of Ti added to the tissue samples.

Additional samples of control animals were used for determination of the LOD. The limit of detection (LOD; as 3x STD, n=20) was estimated on the results obtained for around 0.5 g tissue material from control animals after applying the complete procedure of digestion and measurement with HR-ICPMS. The LOD is 0.05 µg Ti /g tissue.

In conclusion, a suitable method was developed for the determination of Ti in biological tissue and blood samples.

4. Control materials

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To check the accuracy of the analytical method, as no certified reference material exists, two control materials (Seronorm Trace Elements Whole Blood; from Sero AS, Norway) were pretreated and analyzed according to the same procedure in various series. It should be noted that the Ti values in these control sample were presented as additional analytical values and cannot be considered certified reference values. However, they are useful as control samples as they provide an independent value for their Ti content. There is a good agreement between all results (Table 7).

Table 7. Determination of Ti in two commercially available control materials

Sample [a]	Reference concentration provided as "additional analytical values"	Measurement results
L-2 LOT 1003129	18 ± 5 µg/L Ti 19.5 ± 3.1 µg/L Ti	19 ± 6 µg/L Ti (n=11)
L-3 LOT 1112691	12.8 ± 0.4 µg/L Ti	13 ± 4 µg/L Ti (n=9)

a. Seronorm Trace Elements Whole Blood, Sero AS, Norway.

3.2.3 Measurement of tissue samples after in vivo treatment.

The tissue samples were digested with a mixture of nitric acid and hydrofluoric acid (approximately 0.5 g sample + 0.5 mL ultrapure water + 1 mL HNO_3 + 0.75 mL HF) and the total amount of Ti was determined by HR-ICPMS.

The results of the Ti determination in the organ samples are presented in Table 8. It was found that Ti could be detected in several organs at day 14 after intravenous administration of TiO_2 nanomaterial. Aliquots of the same homogenized organs (designated series A for Philips Innovation Services) were sent to ANSES (series B), BfR (series D), and INERIS (series E) for Ti content determination.

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Table 8. Results of Ti tissue levels after intravenous administration of TiO_2 in male rats.

Tissue	Nanomaterial NM-100 Ti level (µg/g tissue)	Sample	Nanomaterial NM-102 Ti level (µg/g tissue)
Liver 2A	103 ± 10 (n=2)	Liver 12A	122 ± 3 (n=2)
Liver 3A	115	Liver 13A	120
Lung 2A	6.4	Lung 12A	19
Lung 3A	4.5	Lung 13A	0.3
Spleen 2A	125	Spleen 12A	65
Spleen 3A	124	Spleen 13A	55
Heart 2A	0.1	Heart 12A	0.1
Heart 3A	<0.1[a]	Heart 13A	0.1
Kidney 2A	<0.1	Kidney 12A	0.1
Kidney 3A	0.1	Kidney 13A	0.1
Brain 2A	<0.1	Brain 12A	<0.1
Brain 3A	0.6	Brain 13A	<0.1
Muscle 2A	0.1	Muscle 12A	<0.1
Muscle 3A	<0.1	Muscle 13A	<0.1

a. Limit of detection in this series of samples was 0.1 µg/g tissue. In additional studies the LOD was lowered to 0.05 µg/g tissue.

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4. ANSES

4.1 Methods

4.1.1 Ti detection by Q-ICP-MS

The measurements were first performed with a Q-ICP-MS (XSeries 2, Thermo Fisher Scientific, Courtaboeuf, France), using ^{71}Ga as internal standard.

Ti consists in total of five naturally abundant isotopes (^{46}Ti, ^{47}Ti, ^{48}Ti, ^{49}Ti and ^{50}Ti). Many studies carried out on various matrices (environmental, biological, and others) found that ^{47}Ti and ^{49}Ti are the less interfered isotopes concerning the analysis with HR-ICP-MS (see section 2). The other three isotopes (^{46}Ti, ^{48}Ti, and ^{50}Ti) cannot be used for quantification by ICP-MS due to isobaric and polyatomic interferences. As example, ^{50}Ti cannot be analysed due to the isobaric interference with ^{50}Cr and ^{50}V. (See Table 1a and 1b in Annex I).

To confirm this information, deionised water solutions spiked with Ti and different non target metallic and major elements at different concentrations were first analysed (see Annex I). Then, biological matrices were spiked with only Ti. In this step, three reference materials of lyophilized bovine liver were used to evaluate the accuracy of Ti analyzed by ICP-MS in biological matrix. The first one is a CRM (NIST 1548a[4]) containing an informative value of Ti (4.7 mg/kg), the other ones (NIST 1577a and BCR 185R from IRMM[5]) were spiked with 5 mg Ti/kg.

Ti was extracted by a microwave digestion under pressure procedure adapted from a validated method for multi elements by ICP-MS (Noël et al., 2003). Several mixtures of HNO_3/H_2O with a final volume equal to 4 mL were tested using two different digestion programs. The best recovery results were obtained with the following microwave digestion program: step 1: 10 min at 800 W; step 2: 10 min at 1000 W, with a sample mass of 0.2 g and a mixture of 3/1 (v/v) of HNO_3 /H_2O (Table 9).

The results indicated that ^{47}Ti and ^{49}Ti were the elements that may not be interfered in water. However, the isotope ^{47}Ti is highly interfered in biological matrices (Table 9) unlike ^{49}Ti. So, only ^{49}Ti was used for the Ti determination by ICP-MS.

[4] National Institute of Standards Technology
[5] Institute of Reference Materials and Measurements

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Table 9: Study of Ti extraction from biological matrix (bovine liver)

	Digestion solution (mL)		Recovery % (Ti measured / Ti spiked) RSD% (n = 3; in brackets)			
Reference materials	HNO_3	H_2O	^{47}Ti	^{48}Ti	^{49}Ti	^{50}Ti
NIST 1577c spiked (5 mg/kg)	2	2	-170 (-224)	92 (31)	**98** (19)	**97** (22)
NIST 1577c spiked (5 mg/kg)	2.7	1.3	26 (220)	97 (11)	**97** (10)	**97** (9)
NIST 1577c spiked (5 mg/kg)	3	1	-51 (-306)	96 (12)	**99** (8)	**101** (8)
NIST 1577c spiked (5 mg/kg)	3.5	0.5	220 (41)	106 (7)	**105** (7)	**108** (7)
NIST 1548a (4.7 mg/kg) Informative value	3	1	309 (7)	256 (3)	**80** (6)	**87** (6)
BCR 185R spiked (5 mg/kg)	3	1	511 (26)	111 (3)	**106** (3)	**100** (3)

4.1.2 Digestion and quantification by ICP-MS of Ti from TiO_2 manufactured nanoparticles (MNs) in different biological matrices

Samples were digested using a Multiwave 3000 microwave digestion system (Anton-Paar, Courtaboeuf, France), equipped with a medium-throughput rotor with 16 medium-pressure vessels made of PEEK and PTFE-TFM (100 mL). This system combines the benefits of a temperature- and pressure-controlled closed-vessel system with rapid microwave heating.

Without available Certified Reference Materials (CRM) containing TiO_2 MNs, the optimisation of the digestion method was first studied using only pure TiO_2 MN (Anatase, 34 nm, VWR), a more difficult matrix compared to biological matrix. Then, the digestion program was optimized using biological Internal Reference Materials (IRMs) spiked with various TiO_2 MNs and at different concentrations (see Table 10).

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Table 10: IRMs used for quality control, analysed by Q-ICP-MS XSeries 2

Biological matrix	Measured total Ti content in spiked samples (mg/kg)	Measured Ti in control samples (mg/kg)	Measured Ti (spiked – control) (mg/kg)**	Theoretical Ti spiked (mg/kg)	Recovery (%): measured/theoretical
Calf heart	**0.65** (RSD* = 3%)	0.51	0.14	0.17	82
Bovine kidney	**2.31** (RSD = 2%)	1.19	1.12	1.26	89
Calf liver	**4.22** (RSD = 8%)	0.89	3.33	3.80	88
Calf muscle	**4.40** (RSD = 2%)	0.52	3.88	4.19	93
Calf brain	**7.57** (RSD = 2%)	0.42	7.14	7.45	96

* RSD % calculated from 20 measurements

** Measured Ti spiked concentration = Measured total Ti content - Measured Ti content in control samples

4.2 RESULTS

4.2.1 Optimisation of reagent conditions on pure TiO_2 MNs

The Ti content in TiO_2 MNs is known ([Ti] = 60% [TiO_2]) and this pure matrix has no interference effect. In this step, only the reagents nature and volume were optimized. The existing validated program (Noël et al., 2003) was used by regulating the digestion phase at the maximum power (P = 1400 W, see Figure 2A).

As optimised for Ti extraction, a mixture of HNO_3/H_2O (3/1, v/v) was initially tested and the results showed that TiO_2 MNs cannot be mineralised without HF acid (a white precipitate was observed). So, the volume HF was optimised from 0.1 to 0.8 mL with mixture of HNO_3/H_2O (3/1, v/v). It should be noted that all quartz compartments of Q-ICP-MS X series II (nebulizer, spray chamber…) were changed by PTFE-TFM ones (Kit-HF) due to the use of HF acid.

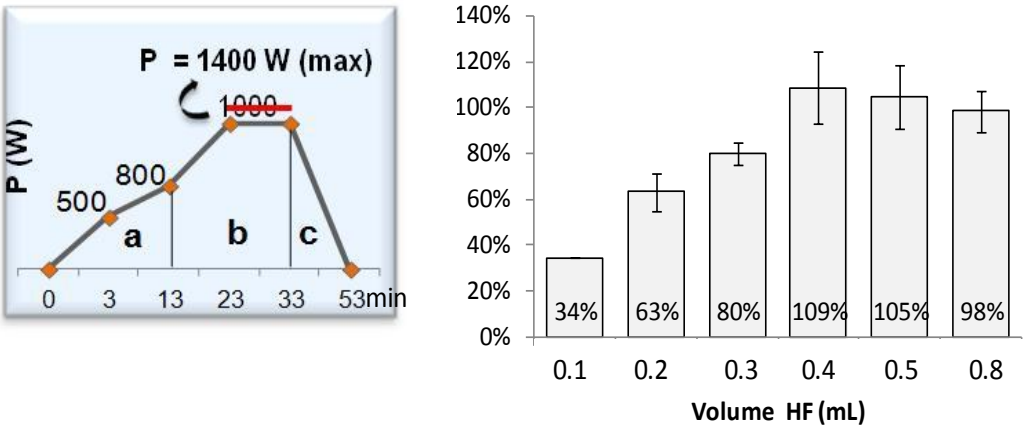

Figure 2. Left (A) digestion program fixed during HF volume optimisation, right (B) recovery obtained (Ti measured/Ti theoretical) for each volume HF tested (n = 2)

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Figure 2B showed that a satisfactory extraction was obtained with a mixture of $HNO_3/H_2O/HF$ at 3, 1 and 0.4 mL respectively.

4.2.2 Optimisation of the microwave program

Based on the optimal reagents ($HNO_3/H_2O/HF$ at 3, 1 and 0.4 mL, respectively) for the Ti extraction, the objective of this step was to optimise the maximum microwave power (initially fixed at the maximum power of the instrument P = 1400 W) and to decrease if possible this power in order to avoid any risk of vessels explosion during the mineralisation.

So, pure TiO_2 MNs, two IRM (calf liver and beef kidney) and beef muscle spiked by TiO_2 dispersion added directly into the pressure vessels (500 - 800 µg TiO_2/g) were used for the microwave power optimisation from 1000 to 1400 W. Results are presented in Figure 3.

It should be noted that the IRM target values were obtained with the maximum microwave power (see 4.1.5 for the preparation of IRMs).

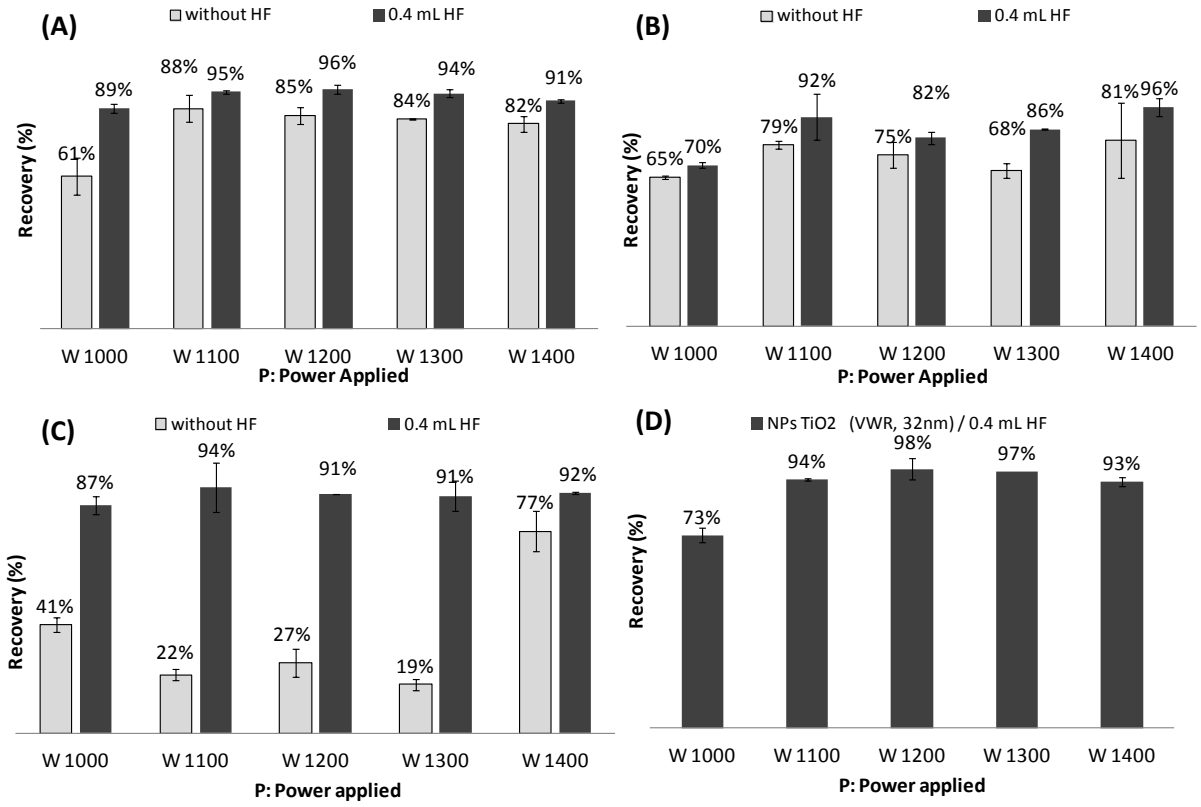

Figure 3. optimisation of the digestion program using (A) beef kidney IRM, (B) calf liver IRM, (C) spiked muscle and (D) pure TiO_2 MNs. Recovery was calculated using target values showed in Table 2: 2.31 and 4.22 µg/g for beef kidney and calf liver IRMs, respectively.

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For all tested matrix, a satisfactory recovery was obtained when the power applied is greater than or equal to 1100 W. So, the P = 1200 W was selected for the mineralisation of TiO_2 MNs present in biological samples.

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In conclusion, the optimised conditions for Ti (from MNs TiO_2) quantification in biological matrices by Q-ICP-MS were:

- **Digestion reagents: $HNO_3/H_2O/HF$ (3/1/0.4 mL)**
- **Microwave programme: 500 to 800 W (10 min) / 800 to 1200 W (10 min) / 1200 W (10 min)**
- **ICP-MS detection: ^{49}Ti isotope with internal standard (^{71}Ga)**

In these optimised conditions, 21 blanks were quantified by Q-ICP-MS X Series 2: the results indicated that the LOQ on ^{49}Ti is about 0.060 µg/g, with a sample mass of 0.3 g and a final volume of 50 mL.

4.2.3 Ti from TiO_2 MNs analysed in Internal Reference Materials (IRM)

The optimised conditions ($HNO_3/H_2O/HF$) (3/1/0.4, v/v/v) and digestion program were used for the determination of Ti in five IRMs (brain, kidney, liver, muscle and heart).
After spiking with a dispersion of TiO_2 MNs, each IRM was distributed in ten pots and Ti was quantified in two aliquots from each pot. The target values is the mean of the 20 measurements (10 pots x 2 replicates), including Ti concentration in the control samples (before spiking) and the added Ti concentration (spiked concentration as TiO_2 MNs), as shown in Table 11.

Table 11: IRMs used for quality control, analysed by Q-ICP-MS XSeries 2

Biological matrix	Measured total Ti content in spiked samples (mg/kg)	Measured Ti in control samples (mg/kg)	Measured Ti (spiked – control) (mg/kg)**	Theoretical Ti spiked (mg/kg)	Recovery (%): measured/theoretical
Calf heart	**0.65** (RSD* = 3%)	0.51	0.14	0.17	82
Bovine kidney	**2.31** (RSD = 2%)	1.19	1.12	1.26	89
Calf liver	**4.22** (RSD = 8%)	0.89	3.33	3.80	88
Calf muscle	**4.40** (RSD = 2%)	0.52	3.88	4.19	93
Calf brain	**7.57** (RSD = 2%)	0.42	7.14	7.45	96

* RSD % calculated from 20 measurements
** Measured Ti spiked concentration = Measured total Ti content - Measured Ti content in control samples

Considering the results in Table 11, recovery calculated confirms the efficiency of the extraction conditions (reagents and digestion program). However, Ti target values were higher than Ti theoretical spiked concentration because of a no negligible and questionable Ti content in the control samples. So, it was interesting to determine whether the origin of Ti measured in control samples was due to interferences or to a natural contamination? Finally, the question arises also the limitation of the use of Q-ICP-MS in standard mode to analyse biological matrices compared to HR-ICP-MS generally used in the literature.

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To answer these questions, four IRMs were analysed before and after spiking, by two others Q-ICP-MS models equipped with a collision cell and a HR-ICP-MS (Figure 4 and Annex II). Results are shown in Table 12.

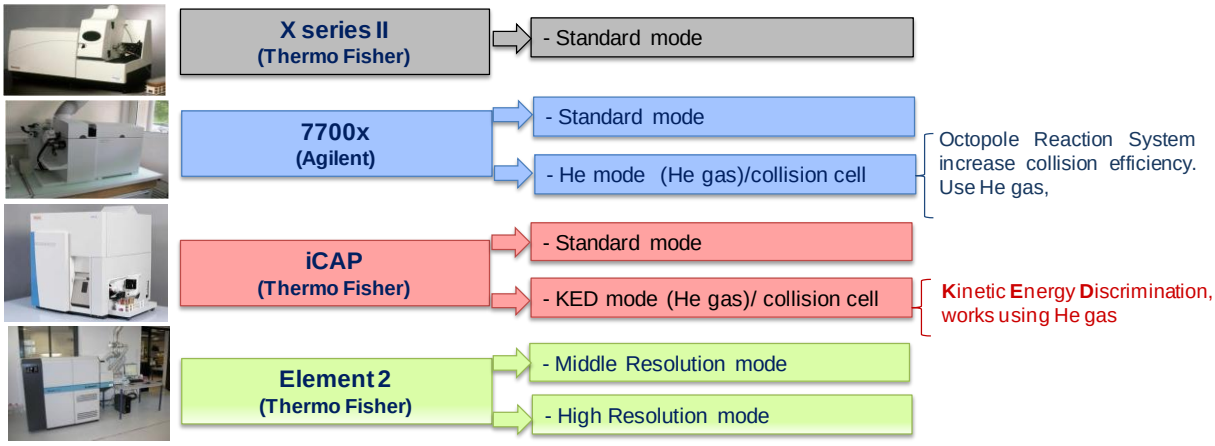

Figure 4. Scheme representing the different ICP-MS and the different modes studied

The LOD and LOQ were also estimated in the Agilent 7700x ICP-MS at 0.05 and 0.10 mg/kg with a sample mass of 0.3 g and a final volume of 50 ML, respectively, but not in the other ICP-MS from Thermo (iCAP and Element 2). For this study, we considered for all ICP-MS devices that the LOQ is similar and equal to 0.10 mg/kg. Results below this LOQ were indicated as < 0.10 mg/kg.

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Table 12: Study comparison between different ICP-MS

Biological matrix	Mode	Measured total Ti in spiked samples (mg/kg)	Measured Ti in control samples (mg/kg)	Measured Ti (spiked – control) (mg/kg)	Theoretical Ti spiked (mg/kg)	Recovery (%): measured/theoretical
Calf Heart (spiked by NM-105)						
XSeries 2 (Thermo)	standard mode	**0.645**	0.509	0.136		80
7700x (Agilent)	standard mode	**0.243**	0.10	0.143		84
	He mode	**0.147**	< 0.10*	0.147	**0.170**	86
iCAP (Thermo)	standard mode	**0.227**	0.10	0.127		75
	He mode	**0.168**	< 0.10	0.168		99
Element 2 (Thermo)	MR mode	**< 0.10**	< 0.10	< 0.10		-
	HR mode	**< 0.10**	< 0.10	< 0.10		-
Bovine kidney (spiked by Anatase TiO_2 NM , 34 nm)						
XSeries 2 (Thermo)	standard mode	**2.31**	1.19	1.12		89
7700x (Agilent)	standard mode	**1.34**	0.12	1.22		97
	He mode	**1.28**	< 0.10	1.28		102
iCAP (Thermo)	standard mode	**1.34**	0.11	1.23	**1.26**	98
	He mode	**1.28**	<0.10	1.32		105
Element 2 (Thermo)	MR mode	**1.31**	<0.10	1.31		104
	HR mode	**1.80**	<0.10	1.18		94

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Table 12: *(continued)*

Biological matrix	Mode	Measured total Ti in spiked samples (mg/kg)	Measured Ti in control samples (mg/kg)	Measured Ti (spiked – control) (mg/kg)	Theoretical Ti spiked (mg/kg)	Recovery (%): measured/theoretical
Calf liver (spiked by Anatase TiO_2 NM, 34 nm)						
XSeries 2 (Thermo)	standard mode	**4.22**	0.89	3.32	**3.80**	88
7700x (Agilent)	standard mode	**3.47**	0.20	3.27		86
	He mode	**3.52**	<0.10	3.52		93
iCAP (Thermo)	standard mode	**3.60**	0.21	3.39		89
	He mode	**3.53**	0.13	3.40		89
Element 2 (Thermo)	MR mode	**3.64**	0.19	3.44		91
	HR mode	**3.95**	<0.10	3.95		104
Calf muscle (spiked by NM-102)						
XSeries 2 (Thermo)	standard mode	**4.40**	0.52	3.88	**4.19**	93
7700x (Agilent)	standard mode	**3.88**	0.12	3.76		90
	He mode	**3.86**	<0.10	3.86		92
iCAP (Thermo)	standard mode	**4.11**	0.11	4.00		95
	He mode	**4.18**	<0.10	4.18		100
Element 2 (Thermo)	MR mode	**4.06**	<0.10	4.06		97
	HR mode	**4.11**	<0.10	4.11		98

Table 12: *(continued)*

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Biological matrix	Mode	Measured total Ti in spiked samples (mg/kg)	Measured Ti in control samples (mg/kg)	Measured Ti (spiked – control) (mg/kg)	Theoretical Ti spiked (mg/kg)	Recovery (%): measured/theoretical
Calf Brain (spiked by NM-100)						
XSeries 2 (Thermo)	standard mode	**7.57**	0.42	7.14		96
7700x (Agilent)	standard mode	**7.75**	<0.10	7.75		104
	He mode	**7.72**	<0.10	7.72		104
iCAP (Thermo)	standard mode	**7.68**	<0.10	7.68	**7.45**	103
	He mode	**7.74**	<0.10	7.94		107
Element 2 (Thermo)	MR mode	**7.76**	<0.10	7.76		104
	HR mode	**8.40**	<0.10	8.40		113

*below the LOQ of 0.10 mg/kg

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For all unspiked matrices (control samples) and for spiked kidney and heart (low Ti concentration), Ti measured by Q-ICP-MS XSeries 2 (standard mode) was interfered, unlike other ICP-MS used. So, this result removes the hypothesis of natural contamination.

The so-called new generation Q-ICP-MS, Agilent 7700x and Thermo fisher iCAP, have no interference whatever the mode used (with or without collision cell) and gives results in good agreement with those obtained by the HR-ICP-MS (except for heart).
However, the interference effect observed with XSeries 2 becomes negligible at high Ti concentration (liver, muscle and brain). Furthermore, the subtraction of the unspiked from the spiked values resulted to comparable Ti concentrations for all tested ICP-MS models and all analyse modes even XSeries 2. Indeed, these results are close to the theoretical spiked Ti values (recovery generally varied between 80 and 113%, except for heart spiked at 2 times the LOQ with iCAP in standard mode (75%).

In conclusion, the Q-ICP-MS Agilent 7700x and Thermo iCAP have no interference whatever the mode used (with or without collision cell) and even if Q-ICP-MS XSeries 2 presents an interference effect, the subtraction of control samples allows overcoming this interference.
This information highlighted the usefulness of using systematically control samples.

4.2.4 Applications of the optimised method on different TiO_2 MNs used for this project

Six different pure MNs studied in the Nanogenotox project were tested (see above Table 1). About 0.03 – 0.10 g of pure TiO_2 MNs were weighed in PTFE-TFM vessels for microwave digestion and mineralized following the optimized digestion program. Prior to the measurement by XSeries 2, the solutions of pure MNs were diluted 10,000 fold. The recovery was calculated considering [Ti] = 60% [TiO_2].

Table 13: Results obtained on six different pure TiO_2 NMs (n = 4)

Sample	Recovery (%)	RSD (%)
Quality control		
IRM calf liver	84	5
IRM bovine kidney	97	3
Spiked bovine muscle	91	5
Pure TiO_2 (anatase, 34 nm, VWR)	92	2
Pure TiO_2 NM-100	95	0
Pure TiO_2 NM-101	81	2
Pure TiO_2 NM-102	86	2
Pure TiO_2 NM-103	76	3
Pure TiO_2 NM-104	89	8
Pure TiO_2 NM-105	93	1

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Results presented in Table 13 show that the optimized method was successfully applied on other TiO_2 NMs which have various properties. It is noted that the low recovery obtained in NM-103 (76%) and NM-101 (81%) can be attributed to the presence of impurities where the Ti concentration should be less than 60% in TiO_2 MNs. Thus, the low sample weight and the high dilution factor should be also a source of error.

4.2.5 Quantification of Ti in organs issued from the acute toxicity test

28 samples consisting of liver, lung, spleen, heart, kidney, brain and muscle issued from rats, intravenously treated with NM-100 or NM-102, were analysed (see above section 2). About 0.1 - 0.5 g of each tissue were weighed in PTFE-TFM vessels for microwave digestion, depending of the amount available. Samples were analyzed once (n = 1) except if there was sufficient sample (n = 3 for liver samples and n = 2 for muscle 3B and 13B).

Quality control

To check the accuracy of the analytical method, the two IRMs (liver and kidney) were digested and analysed with the organs in each experiment.
Furthermore, a Certified Reference Material (CRM) from filtered Lake Ontario water (TM-15.2, HORIBA Scientific, Longjumeau - France) containing Ti (14.6 ± 1.3 µg/L) was systematically used to monitor instrumental drift and to control analytical precision at the beginning and the end of the experiment. This CRM was not digested and it was directly analysed by ICP-MS.
All the results of the IRMs and CRM samples were in the range of 80 - 120% and were considered satisfactory.

Ti level in tissues

Concentrations of Ti present in organs are presented in Table 14. Only liver samples were received as control samples, unfortunately, they have not been subtracted.

Results showed that liver and spleen are the organs with highest Ti concentrations > 100 µg/g, followed by lung with Ti concentrations between 4 and 17 µg/g. With these matrices, whatever the ICP-MS equipment tested and whatever the mode used, all results were comparable and confirmed that the interference effect is negligible at high concentration level.
Concerning the others organs where Ti concentrations were low (heart, kidney, brain, and muscle), Agilent 7700x, iCAP and Element 2 (HR-ICP-MS) gave comparable results. It should be noted that the use of collision cell (He or KED modes) improves slightly Ti determination in some organs. However, the X Series 2 Q-ICP-MS present a very high interference at these low concentrations and the absence of control samples of heart, kidney, brain and muscle does not allow correcting the interfered measurements.
These comparisons between different ICP-MS at high and at low Ti concentration confirm our study conducted on IRMs (as described in 4.2.3 and Table 12) and the importance of control sample to overcome potential interference effects with some ICP-MS devices.

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General conclusions

In this study, an analytical method allowing the **quantification of Ti present as TiO_2 MNs in biological samples was developed**. Ti was extracted from nanoparticles present in the biological samples using microwave digestion technique and a mixture of $HNO_3/H_2O/HF$. Then, the extracted Ti was quantified by ICP-MS using ^{49}Ti isotope.

Studies conducted on controlled matrix spiked by TiO_2 MNs, IRMs, showed that Q-ICP-MS instruments are able to quantify Ti even at low concentrations level (about 0.10 µg/g) without presence of interference effect. These information indicate that the use of HR-ICP-MS and/or collision cell is not obligatory for these biological matrices. It should be noted that unlike other tested ICP-MS, the XSeries 2 model present significant interference in standard mode at low concentrations level but some results on IRMs demonstrated that the subtraction of control samples allows overcoming the interference effect.

Finally, the developed method was applied successfully on organs issued from rats exposed to TiO_2 MNs. Results showed that liver and spleen contained the highest Ti concentrations > 100 µg/g, followed by lung where Ti concentrations varied between 4 and 17 µg/g. All the results are comparable with those obtained by Philips Innovation Services on a HR-ICP-MS, except 3 samples where results obtained by Philips Innovation Services seems overestimated for 3B-Brain or underestimated for 13B-Lung and 12B-Muscle, comparing to those obtained by ANSES on different devices (except X Series 2), including a HR-ICP-MS. These differences may be due to the inhomogeneity of these samples, and/or differences in sample preparation between the laboratories.

Acknowledgments

We would like to thank Dominique Debellis from Thermo Fisher Scientific (Courtaboeuf, France) for his help and for allowing us to carry out tests on iCAP and Element 2 ICP-MS.

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Table 14: Ti levels in various rat organs determined by different ICP-MS.

NM-100 TiO2 with a size of 200-220 nm and anatase crystalline form, Ti level (µg/g tissue)							
ICP-MS model	**X Series 2 (Thermo)**	**7700x (Agilent)**		**iCAP (Thermo)**		**Element 2 (Thermo)**	
Analyse mode	**STD mode**	**STD mode**	**He mode**	**STD mode**	**KED mode**	**MR mode**	**HR mode**
Liver 2B	120 ± 13*	113 ± 3	122 ± 9	121	117	118	121
Liver 3B	120 ± 9	103 ± 3	109 ± 11	103	99	104	99
Lung 2B	8.2	6.2	7.0	7.0	7.2	7.5	6.8
Lung 3B	5.5	4.1	4.4	4.5	4.4	4.5	4.6
Spleen 2B	155	141	148	153	156	158	163
Spleen 3B	136	125	124	130	130	133	129
Heart 2B	1.4	0.27	0.13	0.26	0.12	0.13	<0.10
Heart 3B	1.6	0.20	0.19	0.26	<0.10	0.29	<0.10
Kidney 2B	1.3	0.13	<0.10	0.17	<0.10	<0.10	<0.10
Kidney 3B	1.0	<0.10	<0.10	0.16	<0.10	<0.10	<0.10
Brain 2B	0.9	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
Brain 3B	0.6	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
Muscle 2B	1.2	0.12	<0.10	0.24	<0.10	<0.10	<0.10
Muscle 3B	1.0 ± 0.02	<0.10	<0.10	0.19	<0.10	<0.10	<0.10
LOQ µg/g	0.06	0.10	0.10	nd**	nd	nd	nd

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Table 14. *(Continued)*

NM 102 TiO2 with a size of 15 25 nm and anatase crystalline form, Ti level (µg/g tissue)							
ICP-MS model	**Xseries II (Thermo)**	**7700x (Agilent)**		**iCAP (Thermo)**		**Element 2 (Thermo)**	
Analyse mode	**STD mode**	**STD mode**	**He mode**	**STD mode**	**KED mode**	**MR mode**	**HR mode**
Liver 12B	120 ± 9*	111 ± 7	114 ± 9	115	112	116	112
Liver 13B	120 ± 6	108 ± 4	110 ± 2	113	114	111	115
Lung 12B	17	15	14	16	15	16	16
Lung 13B	15	12	13	14	15	14	14
Spleen 12B	65	58	60	63	63	64	66
Spleen 13B	53	46	49	47	48	49	46
Heart 12B	1.3	0.17	0.21	0.25	<0.10	<0.10	<0.10
Heart 13B	1.5	0.25	0.12	not analyzed			
Kidney 12B	1.0	0.12	<0.10	0.23	<0.10	<0.10	<0.10
kidney 13B	1.0	0.10	<0.10	not analyzed			
Brain 12B	0.7	<0.10	<0.10	0.19	<0.10	<0.10	<0.10
Brain 13B	0.7	<0.10	<0.10	not analyzed			
Muscle 12B	1.4	0.43	0.39	0.64	0.50	0.54	0.42
Muscle 13B	1.0 ± 0.1	0.11 ± 0.04	<0.10	not analyzed			
LOQ µg/g	0.06	0.10	0.10	nd**	nd	nd	nd

* mean + SD (n= 3 for liver and n= 2 for muscle); ** not determined

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5. BfR

5.1 Methods

5.1.1 Digestion method

Approximately 200 mg of the tissue samples were incubated in a capped vial with a total volume of 6 ml consisting of 2 ml conc.HNO_3 and 4 ml 5% HF in 50 ml polypropylene tubes. Samples were digested using a block heater (DigiPrep Jr. digestion system (S-Prep GmbH, Überlingen, Germany), equipped with a heating block for 24 vessels made of low metal content polypropylene (50 ml).

The samples were incubated for 20 minutes at 40°C followed by a stepwise temperature increase of 10°C to a final temperature of 70°C at which the samples were digested for a period of 30 hours. Subsequently, ultrapure water and the internal standard ^{74}Germanium were added to a total volume of 50 ml.

With the applied procedure, it was possible to achieve a good digestion of the original TiO_2 NPs as well as of the biological materials. For some tissues like liver the samples were diluted further.

5.1.2 Method of quantification of Ti in organ samples

Ti levels in tissue were analyzed by Low Resolution Quadrupole ICP-MS (Q-ICP-MS, XSeries 2, Thermo Fisher, Germany) using a set-up of collision cell mode (CCT mode). The instrumental operating conditions of LR-Q-ICP-MS (XSERIES 2) set-up are summarized in Table 15. Values were corrected for ^{74}Germanium as internal standard while ^{49}Ti was selected for the calculations of the Ti content in tissues. To check the accuracy of the analytical method, as no certified reference material (CRM) exists, commercially available reference blood sample with the total concentration of Ti was used (Seronorm Trace Elements Whole Blood; from Sero AS Norway). Additionally TiO_2 nanoparticles (NM-103) samples were prepared and analyzed in the same way as the tissue samples.

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Table 15. Sample introduction system and instrumental operating conditions of Q-ICP-MS (XSERIES 2).

Sample introduction system	
Nebulizer	MicroMist Nebulizer 0.4 ml/min; from ESI GmbH, Germany
Operation mode	CCT-Mode 7 % H_2/He (5ml/min)
Spray chamber	Cyclon spray chamber, from ESI GmbH, Germany (Quartz)
Cones	Nickel

Instrumental operating conditions	
RF power	1500 W
Cool gas flow	13.02 L/min Ar
Auxilary gas flow	0.7 L/min
Sample gas flow	0.97 L/min

5.1.3 Quality control experiments

1. Determination of presence of Ti in polypropylene tubes used and related correction of the raw data
2. Analysis of independent duplicate samples from original TiO_2 dispersions
3. Control materials (Seronorm Trace Elements Whole Blood; Sero AS Norway (L-2 LOT 1003192 with values of 18 ± 5 µg Ti/L and 19.5 ± 3.1 µg Ti/L)

5.2 Results

5.2.1 Determination of presence of Ti in polypropylene tubes

Due to the fact that Ti can be also abundant in polypropylene tubes and in the used chemicals (acids and water), it is of great relevance to determine the blank concentration of Ti and to correct the raw analytical data for this concentration of Ti. The blank concentration of Ti was determined for 50ml ml polypropylene tubes from the selected supplier and the result is given in Table 16.

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Table 16. Blank concentration of Ti concentrations from 50 ml polypropylene tubes and the used chemical

Supplier	c(Ti) [µg/L]
DigiPrep Tubes, Überlingen	0.007 ± 0.009 (n=5)

During this study, in all series tubes from DigiPrep Tubes, Überlingen (S-Prep GmbH, Überlingen, Germany) were used and regularly controlled.

5.2.2 Control materials

To check the accuracy of the analytical method, as no certified reference material exists, a control materials (Seronorm Trace Elements Whole Blood; Sero AS, Norway) was pretreated and analyzed according to the same procedure in various series (Table 17).

Table 17. Determination of Ti in two commercially available control materials

Sample [a]	Reference concentration provided as "additional analytical values"	Measurement results
L-2 LOT 1003192	18 ± 5 µg/L Ti 19.5 ± 3.1 µg/L Ti	19 ± 7 µg/L Ti (n=4)

a. Seronorm Trace Elements Whole Blood, Sero AS, Norway

The analysis of 4 chemical blanks indicated that the limit of quantification (LOQ) on ^{49}Ti is about 0.025 µg/g with a sample mass of 0.2 g. This equals a limit of detection LOD) of approximately of 0.01 µg Ti/g tissue (calculated for 0.2g tissue). In conclusion, a suitable method was developed for the determination of Ti in biological tissue and blood samples.

5.2.3 Measurement of tissue samples after in vivo treatment

The results of the Ti determination in the organ samples are presented in Table 18. It was found that Ti could be detected in several organs at day 14 after intravenous administration of TiO_2 nanomaterial.

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Table 18. Ti levels in various rat organs as determined by ICP-MS.

Tissue	Nanomaterial NM-100 Ti level (µg/g tissue)	Sample	Nanomaterial NM-102 Ti level (µg/g tissue)
Liver 2D	105 ± 7 (n=2)	Liver 12D	124 ± 5 (n=2)
Liver 3D	133 ± 2 (n=2)	Liver 13D	118 ± 2 (n=2)
Lung 2D	6.2	Lung 12D	17
Lung 3D	6.6	Lung 13D	17
Spleen 2D[a]	245	Spleen 12D	101
Spleen 3D[a]	179	Spleen 13D	113
Heart 2D	0.8	Heart 12D	1
Heart 3D	0.8	Heart 13D	0.8
Kidney 2D	0.7	Kidney 12D	0.6
Kidney 3D	0.7	Kidney 13D	0.6
Brain 2D	0.5	Brain 12D	0.7
Brain 3D	0.6	Brain 13D	0.8
Muscle 2D	0.7 ± 0.1 (n=2)	Muscle 12D	0.7
Muscle 3D	0.6 ± 0.1 (n=2)	Muscle 13D	0.6

a) For the spleen only small tissue samples were available, so, low sample weights had to be used. In all cases the values were below 100 mg in one case even below 40 mg. For the Ti determinations samples below 100 mg are generally not sufficient. The digestion method used, was validated for sample weights above 100 mg. In addition, due to local restrictions in these experiments concentrated HF could not be applied, so a lower quality of HF was used. As a consequence the background level of isobaric interferences for these samples were much higher than for all the other samples as no further dilution steps could be applied. For samples with a higher weight the interference effect of the lower quality HF is not a problem in view of the dilutions applied.

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6. INERIS

This part presents INERIS's protocol of preparation and analysis of nanoparticles of titanium dioxide (TiO_2) in biological organs from rats (liver, kidney, lung, spleen, heart, muscle, brain) by Inductively Coupled Plasma Mass Spectrometry (Q-ICP-MS AGILENT 7500).

6.1 Methods

6.1.1 Digestion method

For digestion procedures, about 0.5–1.5 g of each tissue were weighed in high pressure teflon vessels for microwave digestion, 2 ml HNO_3 (suprapure grade, Analytica) were added and samples left to stay for about 12h. Then 0.2 ml HF (suprapure grade, Analytica) was additionally added and samples were digested in a microwave assisted digestion system (CEM - MARS X-PRESS) according to the following program: step 1: 45 min up to 185°C; step 2: 20 min at 185°C. After cooling, to neutralized HF, 2 ml H_3BO_4 (boric acid at 55 g/L) was added to the solutions and samples were placed in a microwave assisted digestion system according to the following program: step 1: 20min up to 160°C; step 2: 10 min at 160°C.
After cooling the solutions were quantitatively transferred to plastic tubes and diluted to 20 ml with MiliQ water.
Before analysis, all samples were diluted by 10 to preserve the ICP-MS from the acid matrix (HNO_3-HF-H_3BO_4). Samples with lower weight were only diluted by 5 (lung, spleen and heart) and very light samples were not diluted (kidney, brain, muscle and lymph nodes).

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6.1.2 Optimization of measurement

6.1.2.1 Determination of best isotopes

The 5 isotopes most abundant of titanium are listed in Table 19 below.

Table 19: Abundance and interference of some isotopes of titanium

Isotope	Isotopic abundance (%)	Interferents (*)
^{46}Ti	7.95	SN, NO_2, N_2O, Ca, SiO, CO_2, Zr^{++}
^{47}Ti	7.75	NO_2, PO, SiO, CCl, SNH, SiOH, SN, N_2, NO_2H, Zr^{++}
^{48}Ti	73.45	ArC, CCl, SO, NO_2, PO, SN, NN_2, C_4, Ca, Zr^{++}
^{49}Ti	5.51	SOH, CCl, PO, SO, CaH, HSO, ClN, SN, NO_2H, NCl, ArC, ArCH,
^{50}Ti	5.34	Cr, V, ArC, CCl, HSO, SO, ArN, ClN, SN, HNCl

* sources: NF EN17294 standard, May and Wiedmeyer 1998, ICP-MS Logiciel Perkin-Elmer

The most abundant isotope ^{48}Ti should allow reaching the lowest limit of quantification but carbon, nitrogen and argon could interfere with the results when present in the organic matrix. After some tests on spiked organs (liver especially), best results were obtained on isotope ^{46}Ti and on ^{49}Ti (see results in Table 20). The analysis have been done with both the isotopes ^{46}Ti and ^{49}Ti showing good consistency between results. The possible isobaric interference of ^{46}Ca indeed does exist, but the natural abundance of ^{46}Ca is very low at 0.003%, and this interference was directly subtracted by the software.

The measurements were performed with five calibration ranges and analyzed under conditions of intermediate precision for the two isotopes used (^{46}Ti and ^{49}Ti). Using various TiO_2 spiked organs (liver especially), the best results were obtained on isotope ^{46}Ti and on ^{49}Ti (Table 20). Indeed ^{50}Ti was excluded and regarding the others, choice was in favor of ^{46}Ti and ^{49}Ti due to the result on brain matrix. Organs were spiked with a dispersion of NM-105 TiO_2 at concentrations ranging from 25 to 500 mg/L of TiO_2. The dispersions were homogenized by ultrasonication before adding to the organ samples.

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Table 20: Recovery (%) of TiO_2 from different organs spiked with TiO_2 solution.

	^{46}Ti	^{47}Ti	^{48}Ti	^{49}Ti	^{50}Ti	TiO_2 (µg/L)
lung	99	101	103	98	276	24
spleen	114	114	113	117	156	276
heart	108	107	108	107	305	4
brain	118	120	122	113	150	8
muscle	85	92	89	84	286	10
kidney	104	109	110	104	193	8
liver	101	103	102	98	111	2270
Average	104	106	107	103	211	

6.1.2.2 Choice of internal standard

An internal standard (IE) is used to limit the impact of the matrix on the results by analysis of an element at the same level of concentration in each sample and standard. Three internal standards were tested: ^{159}Tb (terbium), ^{115}In (indium) and ^{71}Ga (gallium). The stability of internal standard responses (Ga, In, and Tb) is followed along an analytical sequence with and without collision cell. The stability of the three internal standards was determined and is presented in Figure 5.

Ga was excluded because of instability of the analytical response for this isotope. The choice between In and Tb was clearly not evident and only based on the laboratory experience which routinely used Tb as internal standard.

On a liver spiked at 0.5 and 1 ppb, recoveries were similar with the three internal standards without the cell collision. In cell collision mode, recoveries of 137% and 187% are observed with ^{115}In and ^{71}Ga as internal standard respectively. Recoveries of 107% and 133% are observed with the ^{159}Tb as internal standard. Without internal standard, the recoveries of 135% and 169% are observed.

Using of internal standard is required and the terbium was used as the internal standard for this study.

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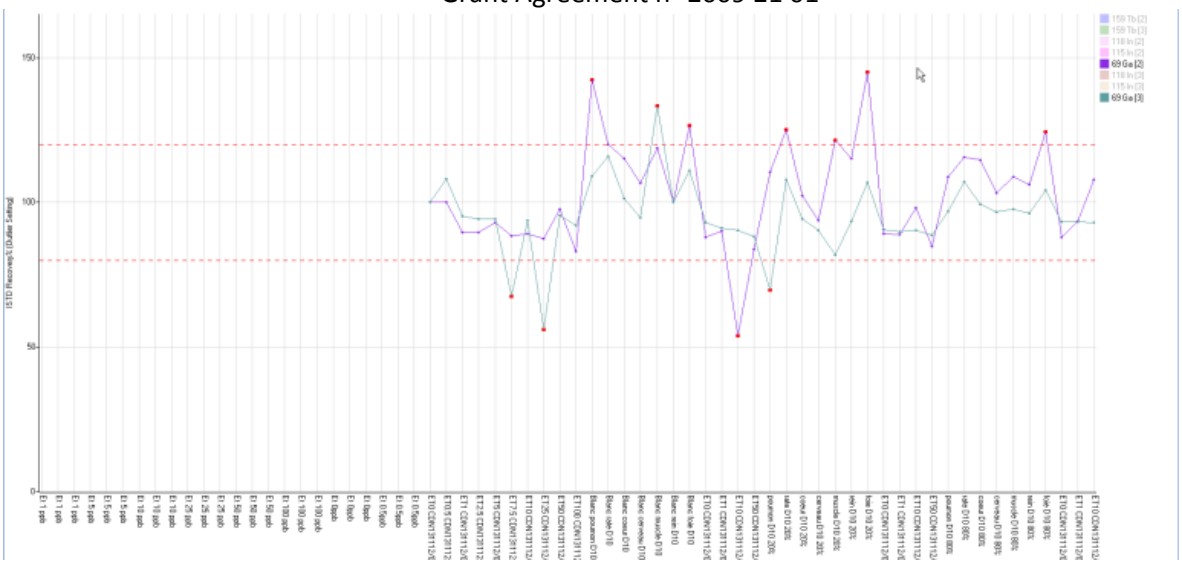
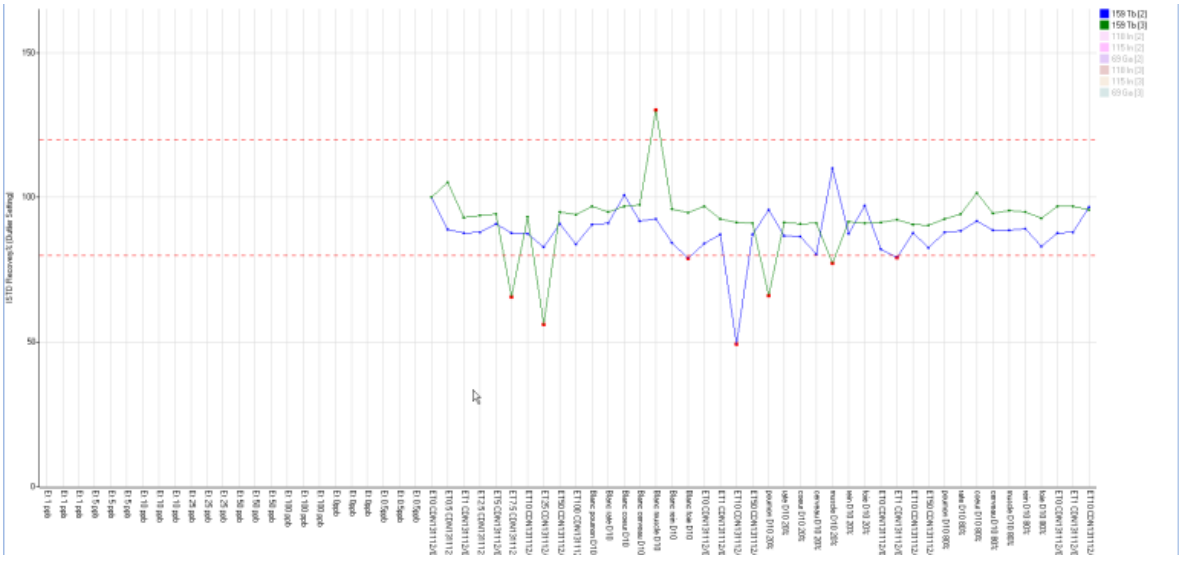


Stability of ^{71}Ga (gallium)



Stability of ^{159}Tb (terbium)

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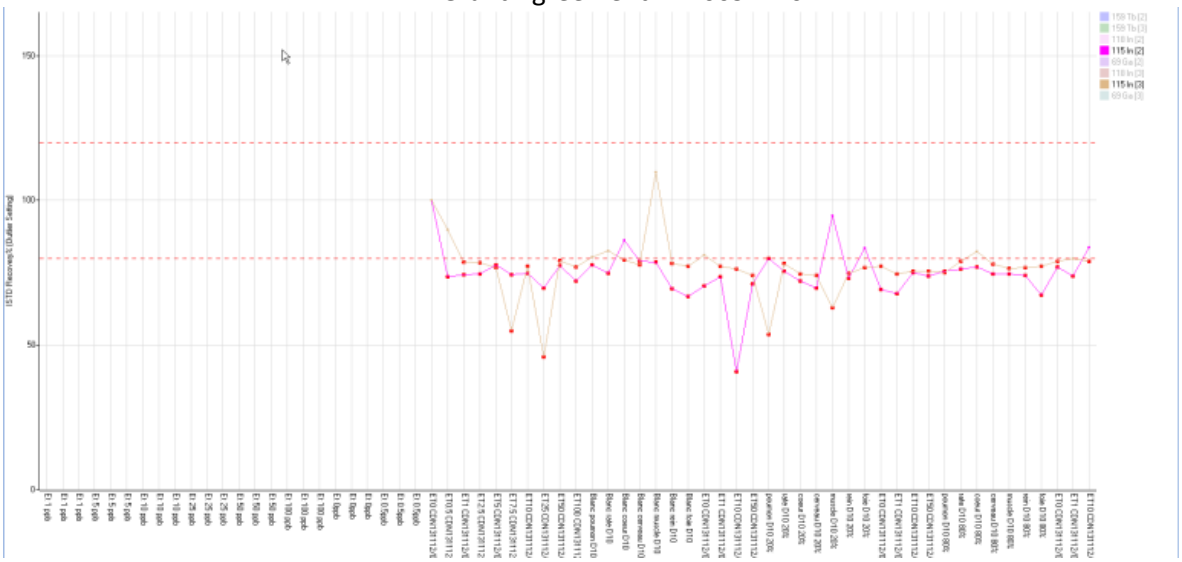

Stability of ^{115}In (indium)

Figure 5. Stability of internal standards.

6.1.4 Limit of quantification

For each organ and selected isotopes, the limit of quantification (in µg/L) has been validated according to the standard NF T 90-210. Limits of quantification were determined from spiked organs at 1µg/L and 2.5µg/L, based on in house laboratory experience. These samples have been analyzed in 5 different calibration series with 2 repetitions per series. The limit of quantification was verified with a maximum acceptable deviation which is formally suggested by the standard at 60%. The limit of quantification is presented in Table 21.

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Table 21: Limit of quantification for each organs and isotopes (µg/L)

Organs	^{46}Ti	^{49}Ti
Liver	1	2.5
Spleen	2.5	2.5
Heart	2.5	2.5
Muscle	2.5	2.5
Kidney	2.5	2.5
Brain	2.5	2.5
Lung	2.5	2.5

The limits of quantification of 1 µg/L or 2.5 µg/L are equivalent to 0.4 µg/g or 1.0 µg/g fresh tissue for ideally 0.5 g of tested material and a final volume of 20 mL. In fact, the final volume is fixed but the quantity of the material was variable from 0.05 g (e.g. spleen) up to 1 g (e.g. liver) depending on the organs. For the lower quantity of the organs, the dilution factor was not systematically applied. Then, the final LOQ depended of these conditions of sample preparation.

Conclusions

The optimised conditions for Ti (from MNs TiO_2) quantification in biological matrices by Q-ICP-MS AGILENT 7500 were:

* Digestion reagents: HNO_3 /HF (2/0.2 mL)
* Microwave program: step 1: 45 min up to 185°C; step 2: 20 min at 185°C.
* ICP-MS detection: ^{46}Ti and ^{49}Ti isotope with internal standard (^{159}Tb)

All different organs were be analyzed with the same calibration ranges. Each calibration solution was prepared in reconstituted acid matrix. According to the NF T 90-210 standard, five calibration ranges were prepared independently and analyzed under conditions of intermediate precision for each isotope (^{46}Ti and ^{49}Ti). These calibration ranges for ^{46}Ti and ^{49}Ti are constituted of at least seven different concentration levels. Results obtained are the average of ^{46}Ti and ^{49}Ti and expressed as µgTi/g fresh tissue and µgTi/organ.

The working range of the method was validated from 1 µg/L (or 2.5 µg/L depending on organs) up to 100 µg/L.

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6.1.5 Analytical conditions

Inductively Coupled Plasma Mass Spectrometry (ICP-MS AGILENT 7500) was used for the determination of titanium concentration in the samples solutions (the optimal instrumental parameters are presented in Table 22). Results obtained are calculated and expressed as micrograms Ti or TiO_2 per gram fresh tissue and micrograms Ti or TiO_2 per organ. Calibration solutions (0-1-2.5-5-7.5-10-20-50-100 µg/L) used were prepared in the matrices diluted by 10.

Table 22. Optimal instrumental parameters for ICP-MS measurements.

Parameters	
Power	1550 W
Argon flow :	
Plasma	15 L/min
Carrier	0.85 L/min
Make up	0.2 L/min
Nebulizer	Concentric
Sample uptake	0.4 ml/min
Integration time	0.3 s / mass
Replicates	3
Isotopes	^{46}Ti and ^{49}Ti
Internal standard	Terbium
Gas of the collision cell	Helium (5 ml/min)

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6.2 Results

6.2.1 Quantification of Ti in organ samples

The Ti concentration in organs of vehicle treated control animals as provided by RIVM are presented in Table 23. Results obtained with animals treated with TiO_2 nanomaterials NM-100 and NM-102 are presented in Table 24.

Table 23. Determination of Ti concentration in livers of vehicle treated animals.

Tissue	Vehicle treated
Liver	Ti level (µg/g tissue)
19a	<0.2
20a	0.3
21a	<0.2
22a	<0.3
23a	<0.3
24a	<0.3
37a	<0.2
38a	<0.2
39a	<0.3

The organ samples obtained from RIVM were fractions of approximately 1g.

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Table 24. Ti levels in various rat organs as determined by ICP-MS.

Tissue	Nanomaterial NM-100	Sample	Nanomaterial NM-102
	Ti level (µg/g tissue)		Ti level (µg/g tissue)
Liver 2E	90	Liver 12E	133
Liver 3E	105	Liver 13E	121
Lung 2E	9.2	Lung 12E	19
Lung 3E	4.1	Lung 13E	9.5
Spleen 2E	187	Spleen 12E	66
Spleen 3E	167	Spleen 13E	66
Heart 2E	0.5	Heart 12E	0.6
Heart 3E	0.4	Heart 13E	0.5
Kidney 2E	<0.4	Kidney 12E	<0.4
Kidney 3E	<0.4	Kidney 13E	<0.3
Brain 2E	<0.3	Brain 12E	<0.5
Brain 3E	<0.4	Brain 13E	<0.4
Muscle 2E	<0.3	Muscle 12E	<0.4
Muscle 3E	0.2	Muscle 13E	<0.2

As the quantity of material was variable but the final volume was fixed, kidney, brain and muscle samples were diluted 5 times; lung, spleen and heart samples were not diluted, and liver samples were diluted 10 times. Thus, the final LOQ depended of these conditions of sample preparation.

Additionally measurements were performed on tissue samples of liver and mesenteric lymph nodes (MLN) as provided by NRCWE obtained from animals that were treated orally by gavage for 5 consecutive days with either vehicle, NM-101, NM-102, NM-103, NM-104 or NM-105 (2.3 mg per day per animal). These samples were analyzed only once in view of the limited amount of tissue available. The homogenized mesenteric lymph nodes (MLN) and the gastro-intestinal (GI)-tract have not been included in the validation tests, thus the LOQ is missing. After the digestion, it appeared that the solutions obtained were limpid and the systematic dilution by 10 should be not applied for MLN. Without this dilution factor, the quantification level for the homogenized mesenteric lymph node is lower than the quantification level for the other organs. Nevertheless, in order to verify the results obtained, some samples of lymph nodes have been

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spiked and recovery was higher than 80%. In these additional studies the liver and GI-tract samples were diluted 10 times. The results of these determinations are presented in Table 25.

Table 25. Ti levels in µgTi/g tissue in various rat organs as determined by ICP-MS.

Treatment[a]	Liver	GI –tract[b]	MLN[c]
Vehicle (19)	<0.4	28.5	0.22
Vehicle (20)	<1.0	29.5	0.14
Vehicle (21)	<0.5	16.8	0.10
NM-101 (22)	<0.6	nd	0.15
NM-101 (23)	<0.5	nd	0.14
NM-101 (24)	<0.5	nd	0.11
NM-102 (25)	<0.6	40.6	0.44
NM-102 (26)	<0.7	26.9	0.12
NM-102 (27)	<0.5	35.6	0.16
NM-103 (28)	11.4	nd	0.23
NM-103 (29)	<0.6	nd	0.09
NM-103 (30)	<0.6	nd	0.20
NM-104 (31)	0.55	nd	0.09
NM-104 (32)	<0.5	nd	0.21
NM-104 (33)	<0.4	nd	0.16
NM-105 (34)	<0.6	nd	0.12
NM-105 (35)	<0.6	nd	0.22
NM-105 (36)	<0.6	nd	0.40
Vehicle (37)	<0.7	nd	0.19
Vehicle (38)	<0.4	nd	0.19
Vehicle (39)	<0.3	nd	0.52
NM-101 (40)	<0.2	nd	0.45
NM-101 (41)	0.61	nd	0.32
NM-101 (42)	<0.3	nd	0.49
NM-105 (43)	0.99	nd	0.39
NM-101 (44)	0.32	nd	0.74
NM-101 (45)	<0.3	nd	0.25

a) Animals received oral administrations of TiO_2 nanomaterials by gavage once a day for 5 consecutive days. b) GI-tract, gastro-intestinal tract. c) MLN, mesenteric lymph node.

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7. Evaluation

The overall results for the Ti determination of 28 samples by four different laboratories are presented in Tables 26A and 26B. It was found that using in house digestion procedures, ICP-MS equipment and reagents at four different locations, in general similar results were obtained for the Ti content in various tissue samples (details are summarized in Table 27). There was reasonable agreement when concentration levels were >4 µgTi/g, independent of the equipment and reagents. The exception was one lung sample from a rat treated with NM-102 measured by Philips Innovation Services. Note that spleen BfR measurements were consequently higher that the measurements obtained by the other laboratories, whereas the other measurements were in the same order of magnitude. An explanation might be the non-optimal sample mass used for the digestion method for these particular spleen samples. The spleen samples available were quite small, resulting in a deviation of the developed digestion method that was validated for samples of 100 mg or more.

At the low concentration levels (< 1.5 µg/g), measurements of Philips Innovation Services (using HR-ICP-MS) were similar than those obtained by ANSES using Q-ICP-MS in both standard and He (CCT) modes or lower than those obtained by INERIS and BfR (using Q-ICP-MS and CCT mode). Except for Lung #13 where the Philips method gives a result of 0.3 µg Ti/g largely below the others (range 10-17 µg Ti/g).

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Table 26A. Ti content of organs in µg/g organ as determined in different laboratories.

NM-100 TiO_2 with a size of 200-220 nm and anatase crystalline form

Sample	Animal	Philips-RIVM	ANSES[a]	ANSES	BfR	INERIS
		MR[b]	STD	CCT (He)	CCT (He/H_2)	CCT (He)
Liver	2	103 ± 10 (2)[c]	113 ± 3 (3)	122 ± 9 (3)	105 ± 7 (2)	90
Liver	3	115	103 ± 3 (3)	109 ± 11 (3)	133 ± 2 (2)	105
Lung	2	6.4	6.2	7.0	6.2	9.2
Lung	3	4.5	4.1	4.4	6.6	4.1
Spleen	2	125	141	148	245	187
Spleen	3	124	125	124	179	167
Heart	2	0.1	0.27	0.13	0.8	0.5
Heart	3	<0.1	0.20	0.19	0.8	0.4
Kidney	2	0.1	0.13	<0.10	0.7	<0.4
Kidney	3	0.1	<0.10	<0.10	0.7	<0.4
Brain	2	<0.1	<0.10	<0.10	0.5	<0.4
Brain	3	0.6	<0.10	<0.10	0.6	<0.4
Muscle	2	0.1	0.12 (2)	<0.10	0.7 ± 0.1 (2)	<0.4
Muscle	3	<0.1	<0.10 (2)	<0.10	0.6 ± 0.1 (2)	<0.4

a) Results obtained on Agilent 7700. b) MR Medium Resolution mode used for measurements, STD, standard mode, CCT (He) Collision Cell Technology mode with Helium, He/H_2 CCT mode with Helium and Hydrogen. c) Data are presented as mean ± standard deviation within brackets number of measurements when applicable.

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Table 26B. Ti content of organs in µg/g organ as determined in different laboratories.

NM-102 TiO_2 with a size of 15-25 nm and anatase crystalline form

Sample	Animal	Philips-RIVM	ANSES[a]	ANSES	BfR	INERIS
		MR[b]	STD	CCT (He)	CCT (He/H_2)	CCT (He)
Liver	12	122 ± 3 (2)[c]	111 ± 7 (3)	114 ± 9 (3)	124 ± 5 (2)	133
Liver	13	120	108 ± 4 (3)	110 ± 2 (3)	118 ± 2 (2)	121
Lung	12	19	15	14	17	19
Lung	13	0.3	12	13	17	9.5
Spleen	12	65	58	60	101	66
Spleen	13	55	46	49	113	66
Heart	12	0.1	0.17	0.21	1	0.6
Heart	13	0.1	0.25	0.12	0.8	0.5
Kidney	12	0.1	0.12	<0.10	0.6	<0.4
Kidney	13	0.1	0.10	<0.10	0.6	<0.4
Brain	12	<0.1	<0.10	<0.10	0.7	<0.4
Brain	13	<0.1	<0.10	0.10	0.8	<0.4
Muscle	12	<0.1	0.43	0.39	0.7	<0.4
Muscle	13	<0.1	0.11 ± 0.04 (2)	<0.10	0.6	<0.4

a) Results obtained on Agilent 7700. b) MR Medium Resolution mode used for measurements, STD, standard mode, CCT (He) Collision Cell Technology mode with Helium, He/H_2 CCT mode with Helium and Hydrogen. c) Data are presented as mean ± standard deviation within brackets number of measurements when applicable.

One may explain the differences that were observed also by the different approaches taken by the laboratories for the total procedure of Ti determination. Table 27 shows the procedures used. Besides difference in ICP-MS equipment used in the various laboratories, there were also differences in the actual digestion method regarding the amounts of acids used, heating temperature, and incubation times, and the vials used during the digestion procedure. All these differences may have contributed to the differences seen in the Ti determination of the tissue samples. In addition, the tissue samples were not completely homogenized using a blender, but the tissue was cut in small pieces and homogenized by hand. This procedure may at hindsight

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also have had an effect on the homogeneity of the tissue samples. However, in view of the number of samples obtained in the various studies mechanical homogenization was considered to be not feasible.

Table 27. Procedures used for Ti determination as presented in Tables 26A and 26B.

Laboratory	Digestion technique	Digestion instrumental setting	Digestion Medium	ICP-MS type	ICP-MS settings
ANSES	microwave 10 min (500 to 800 W) Multiwave 100 ml PEEK and PTFE-TFM vessels	 10 min (800 - t0 1200W) 10 min (1200 W)	0.3-0.5 g tissue 3/1/0.4 $HNO_3/H_2O/HF$ final volume 50 mL	Q-ICPMS Standard mode or CCT-mode (He) Agilent 7700x	^{49}Ti ^{71}Ga as IS
BfR	Open digestion Disposable PP 50 ml vials	20 min 40°C stepwise to 70°C for 30 h	0.2 g tissue 2 ml HNO_3 4 ml 5% HF final volume 6 ml	Q-ICPMS with CCT (Thermo Xseries 2)	^{49}Ti ^{72}Ge as IS
INERIS	microwave Teflon vials	12 RT 1. stepwise 45 min to 185°C + 20 min 2. stepwise 20 min to 160°C + 10 min	0.5-1.5 g tissue 2 ml HNO_3 12 h 0.2 ml HF microwave Final volume water 20 ml	ICP-MS (Agilent 7500)	^{46}Ti and ^{49}Ti ^{159}Tb as IS
Philips Innovation Services	Open digestion block heater 15 ml PP vials	stepwise to 90°C for 2 days	0.5 g tissue 0.5 ml water 1 ml HNO_3 0.75 ml HF Final volume diluted 15 ml	HR-ICP-MS (Element XR) MR mode	^{47}Ti ^{115}In as IS

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8. Conclusions

The results obtained for the Ti detection in various organs were in general in agreement with each other when Ti concentrations were >4 µg/g tissue. However, in some spleen measurements were higher compared to the measurements at the other laboratories, and also at the lower concentrations some differences were noted between the four laboratories.

In conclusion, the method for the determination of Ti in tissues was successfully developed, and with some modifications applied in various laboratories. A combination of nitric acid (HNO_3) and hydrofluoric acid (HF) gave the best results for tissue and titanium digestion for preparation of the samples for measurement in the ICP-MS. Despite the differences in methodology and independent of the ICP-MS equipment used similar results were obtained for measurements above 4 µg Ti per gram tissue. The measurement of very low levels may be more critical and further investigations may be required in view of the differences observed between the laboratories.

However, in an additional series of experiments at ANSES one subset of the same samples was evaluated on three different Q-ICP-MS devices and one HR-ICP-MS instrument. The ICP-MS devices (see Tables 12 and 14) gave similar results independent of the instrument used, except for the Q-ICP-MS XSeries 2 which presents an interference effect at low concentration levels in standard mode (CCT not tested). The results indicated that the Q-ICP-MS Agilent 7700x and Thermo iCAP have no interference whatever the mode used (with or without collision cell) and even if Q-ICP-MS XSeries 2 presents an interference effect, the subtraction of control samples allows overcoming this interference. This information highlighted the usefulness of using systematically control samples to overcome potential interference effects with some ICP-MS devices. As this comparison was done with the same subset of samples that had been subject to the same sample preparation method, the differences that were observed between the four laboratories might be also due to local conditions, and sample preparation may have a more pronounced effect on the actual outcome of the measurements than the ICP-MS equipment used.

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10. Annexes Additional studies performed at ANSES

Annex I Evaluation of Ti interferences

Ti solutions were used to assess the possible interfering elements on Ti and the Q-ICPMS performances.

Solutions of deionized water spiked by Ti (5 and 10 µg/L) were analysed with the addition of the well-known interfering elements ^{50}Cr, ^{50}V and ^{48}Ca which potentially interfere with ^{50}Ti and ^{48}Ti (Tables 1a and 1b). These potential interfering elements were spiked individually and at the same time with different high concentrations: 1 to 10 mg/L (Table 2(a)).

Table 1a. Ti isobaric and polyatomic interferences

Isotope	Abundance (%)	Interferences
^{46}Ti	7.99	$^{32}S^{14}N^+$; $^{14}N^{16}O_2^+$; $^{15}N_2\,^{16}O^+$; $^{46}Ca^+$; $^{92}Zr^{2+}$; $^{34}S^{12}C^+$; $^{33}S^{13}C^+$; $^{31}P^{15}N^+$; $^{30}Si^{16}O^+$; $^{29}Si^{17}O^+$; $^{28}Si^{18}O^+$; $^{6}Li^{40}Ar$; $^{10}B^{40}Ar$
^{47}Ti	7.32	$^{32}S^{14}N\,^1H^+$; $^{30}Si^{16}O^1H^+$; $^{32}S^{15}N^+$; $^{33}S^{14}N^+$; $^{15}N^{16}O_2^+$; $^{14}N^{16}O_2\,^1H^+$; $^{12}C^{35}Cl^+$; $^{31}P^{16}O^+$; $^{94}Zr^{2+}$; $^{34}S^{13}C^+$; $^{29}Si^{18}O^+$; $^{36}Si^{11}B^+$; $^{30}Si^{16}O^1H^+$; $^{11}B^{36}Ar$; $^{9}Be^{38}Ar$; $^{7}Be^{40}Ar$
^{48}Ti	73.98	$^{32}S^{16}O^+$; $^{34}S^{14}N^+$; $^{33}S^{15}N^+$; $^{14}N^{16}O^{18}O^+$; $^{14}N^{17}N_2^+$; $^{12}C_4^+$; $^{36}Ar^{12}C^+$; $^{48}Ca^+$; $^{96}Zr^{2+}$; $^{36}S^{12}C^+$; $^{31}P^{17}O^+$; $^{30}S^{18}O^+$; $^{31}P^{16}O^1H$; $^{10}B^{38}Ar$; $^{12}C^{36}Ar$
^{49}Ti	5.46	$^{32}S^{17}O^+$; $^{32}S^{16}O^1H^+$; $^{35}Cl^{14}N^+$; $^{34}S^{15}N^+$; $^{33}S^{16}O^+$; $^{14}N^{17}O_2\,^1H^+$; $^{14}N^{35}Cl^+$; $^{36}Ar^{13}C^+$; $^{36}Ar^{12}C^1H^+$; $^{12}C^{37}Cl^+$; $^{31}P^{18}O^+$; $^{36}S^{13}C^+$; $^{34}S^{15}N^+$; $^{31}P^{17}O^1H^+$; $^{11}B^{38}Ar$; $^{9}Be^{40}Ar$
^{50}Ti	5.25	$^{32}S^{18}O^+$; $^{32}S^{17}O^1H^+$; $^{36}Ar^{14}N^+$; $^{35}Cl^{15}N^+$; $^{36}S^{14}N^+$; $^{33}S^{17}O^+$; $^{34}S^{16}O^+$; $^1H^{14}N^{35}Cl^+$; $^{34}S^{15}O^1H^+$; $^{50}V^+$; $^{50}Cr^+$; $^{33}S^{15}N^1H^+$; $^{33}S^{16}O^1H^+$

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Table 1b. Ca, Cr and V isotopes

Ca isotopes	Cr isotopes	V isotopes
^{40}Ca 96.941	^{50}Cr 4.345 % interfere with ^{50}Ti	^{50}V 0.25% interfere with ^{50}Ti
^{42}Ca 0.647 %	^{52}Cr 83.789 %	^{51}V 99.75%
^{43}Ca 0.135 %	^{53}Cr 9.501 %	
^{44}Ca 2.086 %	^{54}Cr 2.365 %	
^{46}Ca 0.004 % interfere with ^{46}Ti		
^{48}Ca 0.187 % interfere with ^{48}Ti		

Furthermore, in order to evaluate the effect of charged matrix (with different non target metallic and major elements at different concentrations) on Ti determination by ICP-MS, standard solutions containing Ti (2, 5, 10, 20 and 50 µg/L) were spiked to create potential disturbing interferences:

- (i) with multi-elemental solution (Li, Al, Cr, V, Ni, Zn, Co, Cu, Ge, Se, Sc, Mn, Fe, Sr, Mo, and Y) at five increasing levels 10, 20, 40, 80 and 160 µg/L;
- (ii) with solution containing major elements (Ca, K, Mg and Na) at three increasing levels 0.5, 1 and 5 mg/L (Table 2(b)).

For both tests, the response of Ti isotopes was recorded and the ratio between measured Ti and Spiked Ti was studied.

Table 2a: Study of interferences

	Spiked elements in deionized water (µg/L)				Measured Ti / Spiked Ti (%)			
	Ti	Ca	Cr	V	^{47}Ti	^{48}Ti	^{49}Ti	^{50}Ti
Ti + V	10	0	0	5000	102	84	**102**	2206
Ti + Cr	10	0	5000	0	80	67	**81**	35829
Ti + Ca	10	5000	0	0	104	299	**104**	175
Ti + Ca Cr V	5	1000	1000	1000	105	150	**105**	19656
	5	2000	2000	2000	104	213	**103**	38863
	5	5000	5000	5000	101	473	**100**	93763
	5	10000	10000	10000	98	820	**98**	182910

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Table 2b: Study of interferences

Spiked elements in deionized water			Measured Ti / spiked Ti (%)			
Ti (µg/L)	Multi-elem (µg/L)	Ca, Na, K, Mg (mg/L)	^{47}Ti	^{48}Ti	^{49}Ti	^{50}Ti
2	10	0	97	86	**114**	481
2	20	0	99	86	**114**	862
2	40	0	96	83	**111**	1577
2	80	0	94	81	**109**	3017
2	160	0	95	81	**108**	6015
10	10	0	94	79	**96**	166
10	20	0	95	80	**97**	241
10	40	0	94	79	**97**	381
10	80	0	92	78	**95**	657
10	160	0	94	79	**97**	1246
20	10	0	94	95	**95**	132
20	20	0	92	95	**94**	164
20	40	0	94	97	**96**	239
20	80	0	94	96	**95**	379
20	160	0	94	95	**95**	671
2	0	0.5	94	-4	**103**	101
5	0	0.5	95	48	**101**	99
10	0	0.5	97	66	**100**	99
20	0	0.5	97	94	**100**	100
50	0	0.5	101	100	**102**	103
2	0	1	101	98	**116**	108
5	0	1	101	91	**108**	106
10	0	1	106	95	**110**	110
20	0	1	104	112	**107**	107
50	0	1	105	109	**106**	108
2	0	5	105	1055	**126**	115
5	0	5	108	497	**117**	114
10	0	5	110	306	**114**	114
20	0	5	112	214	**115**	116
50	0	5	112	154	**114**	116
2	80	1	105	134	**130**	629
5	80	1	102	101	**110**	251
10	80	1	102	95	**107**	176
20	80	1	106	116	**108**	151
50	80	1	106	112	**108**	128

Results showed that ^{47}Ti and ^{49}Ti were the elements that may not be interfered (Table 2(a) and (b)).

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Annex II:

ICP-MS parameters

The optimal instrumental parameters concerning all ICP-MS used for the determination of titanium concentration in organs are presented in Table 3a.

Table 3a: Optimal instrumental parameters for ICP-MS measurements

	Agilent 7700x	**Thermo iCAP**	**Thermo XSeries 2**	**Thermo Element 2**
ICP-MS parameters				
Power	1400 W	1549 W	1400 W	1300 W
Nebulizer	MicroMist Nebulizer 0.15 mL/min	Concentric nebulizer, paired with peltier-cooled cyclonic spray chamber	Concentric nebulizer, cyclonic spray chamber in PTFE (Kit-HF)	Concentric nebulizer
Plasma argon flow rate	15 L/min	14 L/min	13 L/min	16 L/min
He gas flow (collision cell)	4.3 mL/min	4.2 mL/min	-	-
Nebuliser argon flow rate	0.9 – 1 mL/min	1 mL/min	0.95 – 1.1 mL/min	1.3 mL/min
Sampling and skimmer cones	Nickel	Nickel	Nickel	Nickel
Auxiliary argon flow rate	0.99 L/min	0.79 L/min	0.80 - 0.90 L/min	0.88 L/min
Replicates	3	3	3	3
Isotopes	^{49}Ti	^{49}Ti	^{49}Ti	^{49}Ti
Internal standard	^{71}Ga	^{71}Ga	^{71}Ga	^{71}Ga

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• Contact •

Website:
www.nanogenotox.eu

E-mail:
nanogenotox@anses.fr

Coordinator: French Agency for Food, Environmental and Occupational Health & Safety (ANSES)

27-31, avenue du Général Leclerc
94701 Maisons-Alfort Cedex
France

anses
French agency for food, environmental and occupational health & safety

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This document arises from the NANOGENOTOX Joint Action which has received funding from the European Union, in the framework of the Health Programme under Grant Agreement n°2009 21.
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