18 June 2019

DIRECTA PLUS S.p.A.
Via Cavour, 2
22074, Lomazzo (CO)
Italy

Submission date: **29 May 2019**
Submission number: **AX604814-19**
Decision number: **SUB-D-2114475408-40-01/F**
EC number: **801-282-5**

DECISION ON YOUR REGISTRATION

Based on Article 20(2) of Regulation (EC) No 1907/2006 ('REACH'),

Your registration for the substance with EC number **801-282-5** is complete. This registration entitles you to manufacture/import the substance, or produce or import an article containing it.[1]

Your registration covers:

- the tonnage band **between 1 and 10 tonnes/year**

The registration number is: **01-2120768618-38-0004**

The registration date is: **29 May 2019**

Further observations

In accordance with Article 20(2) of REACH, the completeness check ascertains that all the elements required have been provided. However, this check does not include an assessment of the quality or adequacy of data provided. Such an assessment may occur later in a compliance check.

Also the verification of the eligibility for any claimed fee reductions (for example such resulting from the declared company size) is not part of the completeness check, but it may follow at any time in accordance with Article 13(3) of Fee Regulation (EC) No 340/2008.

In accordance with Article 22(1) of REACH, registrants are on their own initiative required to update their registrations without undue delay with relevant new information (for instance, change in status, substance composition or quantities).

Certain information from this registration dossier will be published without further notice at https://echa.europa.eu/information-on-chemicals.

Should you have any questions please contact ECHA via the contact form at https://echa.europa.eu/contact.

Tiago Pedrosa
Head of Unit A3 - Submission and Processing
Directorate of Submissions and Interaction

[1] Note to registrants in the United Kingdom: The UK has notified its intention to leave the EU. As this decision on your registration only applies within the framework of the EU REACH legislation, your registration risks becoming non-existent once the UK withdrawal from the EU takes effect. ECHA advises you to regularly consult its webpages on the UK's withdrawal at https://echa.europa.eu/uk-withdrawal-from-the-eu. There you will find updated information on the validity of registrations by UK registrants, and on any action that you may need to take to maintain access to the EU market.

04 April 2017

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Submission date: **27 March 2017**
Submission number: **TU785695-82**
Decision number: **SUB-D-2114358775-35-01/F**

DECISION ON YOUR REGISTRATION

Based on Article 20(2) of Regulation (EC) No 1907/2006 ('REACH'),

Your registration for Graphite is complete. This registration entitles you to manufacture/import the substance, or produce or import an article containing it.

Your registration covers:

- the tonnage band **between 10 and 100 tonnes/year**

The registration number is: **01-2119486977-12-0080**

The registration date is: **27 March 2017**

Advice and further observations

In accordance with Article 20(2) of REACH, the completeness check ascertains that all the elements required have been provided. However, this check does not include an assessment of the quality or adequacy of data provided. Such an assessment may occur later in a compliance check.

Also the verification of the eligibility for any claimed fee reductions (for example such resulting from the declared company size) is not part of the completeness check, but it may follow at any time in accordance with Article 13(3) of Fee Regulation (EC) No 340/2008.

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Farcoderm
TESTED WELLNESS

tested by Farcoderm

IN VITRO PRODUCT SAFETY STUDY

IN VITRO EVALUATION OF THE CITOTOXIC POTENTIAL OF A PRODUCT (3T3 NRU ASSAY)

DIRECTA PLUS SPA

PURE G+

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STUDY DESIGN

Title

In vitro product safety study - in vitro evaluation of the citotoxic potential of product (3T3 NRU assay)

Study aim

This study allows to determine the in vitro basal cytotoxicity of test substances using NRU assays and the use of the in vitro data to determine starting doses for in vivo acute oral systemic toxicity tests. The NRU assay is performed in a dose-response format to determine the concentration that reduces NRU by 50% compared to the controls (i.e. the IC50). The IC50 value is used in a linear regression equation to estimate the oral LD50 value (dose that produces lethality in 50% of the animals tested), which is then used to determine a starting dose for acute oral systemic toxicity.
In summary, the in vitro data are used to estimate an IC50 value which in turns is used to predict a LD50 value that can serve as the starting dose for the acute oral toxicity test in vivo. This test is also used for the identification of substances not requiring classification for acute oral toxicity (EURL ECVAM Recommendation, 2013), based on a cut-off of LD50 >2000 mg/kg.
The use of the NRU test method in a weight-of-evidence approach to determine starting doses for these acute oral systemic toxicity tests allows to reduce the number of animals required for the tests, according to the Three Rs (3Rs) principle first described by W. Russell and R. Burch in 1959 (Refinement /Reduction/Replacement), transposed by the European Directive (86/609/CEE) and confirmed in the European Directive (2010/63/UE).
The objective of this assay was to assess quantitatively the effects of the test item on cell survival of BALB/c 3T3 fibroblast culture through the NRU assay.
The NRU assay is based on the cell ability to incorporate and bind the Neutral Red, a vital dye. The NR is a cationic dye that penetrate the cell membrane through a mechanism of non ionic diffusion and that is accumulated in the lysosomes, on matrix anionic sites. Cell and lysosome membrane alterations cause lysosomes fragility and gradual irreversible changes in the cells. These changes induced by xenobiotics determinate the decreasing of RN uptake and lysosome linking. Alive, damaged and dead cells can be discriminated with this method.

Tested product

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Powder of pristine graphene nanoplatelets

Carried out test and experimental model

The following test was carried out:

- Cytotoxicity assay by NEUTRAL RED UPTAKE

For the test execution the following cell model was used:

- Fibroblasts BALB/c 3T3 clone 31

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Experimental protocol

OECD (2010) - OECD Series on Testing and Assessment No. 129: Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests.

Sample preparation and test execution

Tested product, emulsified in Corn oil and diluted in culture medium at scalar concentrations from 20 mg/ml, was added to the wells containing the cells (8 concentrations). Cell exposition to tested product was prolonged for 48 hours. Through this period cells were maintained at 37°C and 5% CO_2.

At the end of incubation period, medium containing tested product was removed; cells were washed with PBS and new culture medium, containing NR; was added to the wells.

Cells were incubated at 37°C and 5% CO_2 for 3 hours.

At the end of this period NR-medium was removed, cells were washed with PBS and a solution to solubilize NR captured by cells. When solubilisation is completed, well plate was red at 540 nm.

Corn oil treated cells are used as negative control. Cells treated with Sodium lauryl sulphate (SDS) scalar concentrations are used as positive control.

Result calculation and interpretation

For each tested product concentration, % cell viability is calculated according to the following

$$\% \text{ CELL VIABILITY} = (OD_{540}\ \textit{treated} * 100)/OD_{540}\ \textit{negative control}$$

Corresponding mortality is calculated by subtraction.

Recorded mortality data are plotted against respective product concentrations to create a dose-response curve. By curve equation **NR50 parameter** (or IC50), ie product concentration that reduces the NRU by 50%, is calculated.

The following formula is used to predict LD50 from the obtained NRU IC50 value:

log LD50 (mg/kg) = 0.372 log IC50 (µg/mL) + 2.024 (ICCVAM, 2006a)

Below is reported the Globally Harmonised System (GHS) classification for the acute toxicity:

Endpoint value	GHS Classification
LD50 ≤ 5 mg/kg	Category 1
5 mg/kg < LD50 ≤ 50 mg/kg	Category 2
50 mg/kg < LD50 ≤ 300 mg/kg	Category 3
300 mg/kg < LD50 ≤ 2000 mg/kg	Category 4
2000 mg/kg < LD50 ≤ 5000 mg/kg	Category 5
> 5000 mg/kg	Unclassified

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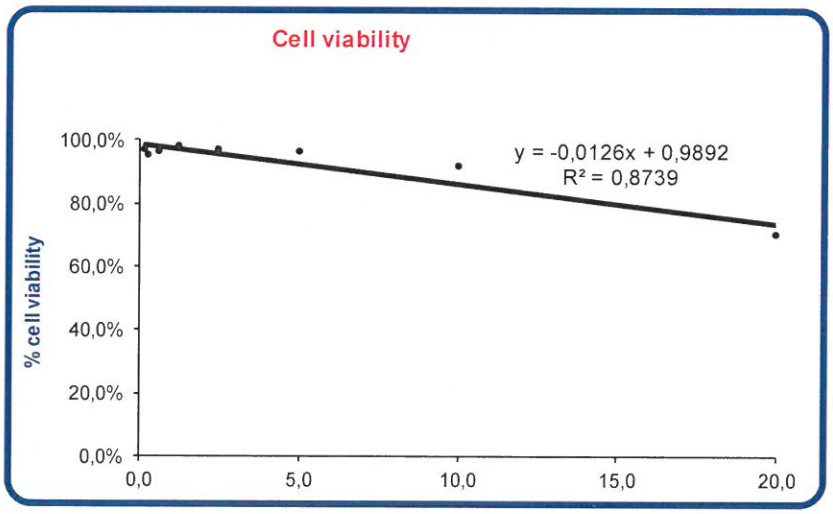
RESULTS AND GRAPHS

TEST ACCEPTANCE CRITERIA

Positive control: NR_{50} of concurrent SLS test	complies
Vehicle control: Corn oil in culture medium	non cytotoxic

TEST RESULTS

Concentration mg/ml	%Cell viability	%Cell mortality
20,00	70,03%	29,97%
10,00	91,89%	8,11%
5,00	96,34%	3,66%
2,50	96,79%	3,21%
1,25	97,73%	2,27%
0,63	96,15%	3,85%
0,31	95,44%	4,56%
0,16	96,87%	3,13%



NR50 CALCULATION

According to the obtained dose-response curve, NR50 is >20 mg/ml
The predicted LC50 value is >2000 mg/kg
The sample is UNCLASSIFIED/NO CATEGORY for acute oral toxicity

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CONCLUSION

The sample named

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Does not show cytotoxic effects on the experimental model.

According to the data obtained from the predictive in vitro model, it is

UNCLASSIFIED/NO CATEGORY for acute oral toxicity.

San Martino Siccomario – 14th January 2016 (first emission)

San Martino Siccomario – 19th April 2016 (new editing)

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BIBLIOGRAPHY

1. DB-ALM protocol n. 139 - BALB/c 3T3 Neutral Red Uptake Cytotoxicity Assay (3T3 NRU).
2. OECD (2010) - OECD Series on Testing and Assessment No. 129: Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests.
3. OECD (2001) - OECD Test guideline No. 423: Acute oral toxicity - Acute toxic class method.
4. EURL ECVAM Recommendation on the 3T3 Neutral Red Uptake Cytotoxicity Assay for Acute Oral Toxicity Testing, 2013

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IN VITRO SAFETY STUDY
BIOLOGICAL EVALUATION OF A MATERIAL
IN VITRO EYE IRRITATION: EPIOCULAR™ EYE IRRITATION TEST (EIT) FOR THE EVALUATION OF OCULAR IRRITATION

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TEST PROCEDURE INFORMATION

Tested product

DIRECTA PLUS SPA

PURE G+

Sample description
Powder of pristine graphene nanoplatelets

Date of test execution
27/01/2016-29/01/2016

Experimental model
EpiOcular™ (MatTek Corporation) batch N° 21591
0.6 cm^2 reconstructed tissue - highly differentiated squamous epithelium of stratified human keratinocytes in a three dimensional structure similar to human corneal epithelium. Cells are grown on inert polycarbonate filter on chemically defined medium, at the air-liquid interface.

Culture medium
MAINTENANCE MEDIUM batch N° 012516MHA

Protocol
Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage (OECD 492)

Positive control
Methyl acetate

Negative control
Deionized water

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SUMMARY

The purpose of the study is the evaluation of ocular irritation of a material by using the alternative method «EpiOcular™ eye irritation test - Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage» (OECD 492). In particular, this method allows to discriminate chemicals not requiring classification for serious eye damage/eye irritancy (UN GHS No Category) from those requiring classification and labelling (UN GHS Categories 1 and 2).
The salient features of this method are reported below:

- study performed on in vitro reconstituted tissue EpiOcular™ MatTek Corporation, a three-dimensional stratified human keratinocytes model stratum corneum free;
- deposit of 50 µL of test material on the surface of tissue for 30 minutes, followed by a 12-minute post-tratment immersion and a 120 minute post-treatment incubation (protocol for liquid substances);
- deposit of 50 mg of test material on the surface of tissue for 6 hours, followed by a 25-minute post-tratment immersion and 18-hour post-treatment incubation (protocol for solid substances);
- assessment of cell viability by MTT method. Validation of the test by positive control (methyl acetate) and negative control (deionized water).

In accordance with the results of cell viability obtained after tissue treatment with test item (94.28), the product

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is classified as NON EYE IRRITANT (UN GHS No Category)

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INTRODUCTION

Study aim

The «EpiOcularTM eye irritation test - Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage» (OECD 492) is designed for the prediction of ocular irritation/corrosion of chemicals by measurement of their ability to produce a decrease in cell viability, as reflected in the MTT assay, on reconstructed human tissue model miming human corneal epithelium. The measurement of viability of the EpiOcular™ RhCE tissue construct after topical exposure to a test chemical to discriminate chemicals not requiring classification for serious eye damage/eye irritancy (UN GHS No Category) from thoserequiring classification and labelling (UN GHS Categories 1 and 2) is based on the assumption that all chemicals inducing serious eye damage or eye irritation will induce cytotoxicity in the corneal epithelium and/or conjunctiva producing tissue damage.

Bibliographic references

This test takes in consideration the following bibliographic references:

MatTek Corporation EpiOcular™ Eye Irritation Test: Protocol for the prediction of acute ocular irritation of chemicals;

OECD Guidelines for the testing of chemicals, OECD 492: Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage, 2015;

INVITTOX method summary n. 164 (EpiOcular™ Eye Irritation Test - Summary).

Tested product

DIRECTA PLUS SPA

PURE G+

Sample description

Powder of pristine graphene nanoplatelets

MATERIALS AND METHOD

Sample and control preparation

Liquid and viscous test substances:

50 µL of the test substance.

Solid test substances:

50 mg of the test substance.

Two tissues per test substance are used.

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Control preparation

Methyl acetate was used as positive control. Deionized water was used as negative control.

Tissues

EpiOcular™ set and media are provided by MatTek Corporation.
Together with the tissues are also provided material quality control that are satisfied: normal histology (absence of significative alterations), cell viability (MTT OD > 0.8), barrier function integrity (4.00 < ET50 < 9.00 hrs), absence of bacteria, fungi, HIV, and Hepatitis B, C.

Test procedure for MTT test

Pre-incubation step

An appropriate number of 6-well plates was filled with 1 mL of fresh maintenance culture medium.
Using sterile forceps, tissues were taken off from agarose and the bottom of the insert was cleaned on sterile absorbent paper to remove eventual remaining agarose pieces.
The tissues were transferred on fresh medium and placed at 37°C, 5% CO_2, 95% RH incubator overnight.

Pre-treatment step

After the overnight incubation, the tissues were pre-wetted with 20 µL of saline phosphate buffer (PBS) and incubated at standard culture conditions for 30 ± 2 minutes.

Sample application: treatment for solid and liquid substances

Test was performed in double: 2 wells per test substance, 2 for positive control and 2 for negative control for each experimental condition

Liquid and viscous test substances:

50 µL of the test substance are applied on the top of each epidermis tissue using micropipette for 30 ± 2 minutes.

Solid test substances:

50 mg of the substance are applied to the epidermis surface for 6 hours ± 15 minutes.

Rinsing and drying steps

Tissues were rinsed with PBS to remove all residual test substance from the epidermal surface. Insert bottom was dried on a sterile absorbent paper. The surface of the tissues was dried with sterile cotton tip.

Post-Soak step

After the rinsing the tissues are immersed in 5 mL of pre-warmed assay medium in a 12-well plate for a 12 ± 2 minutes immersion incubation at room temperature for tissues treated with liquid substances and 25 ± 2 minutes for sample treated with solid substances. This step is intended to remove any test article absorbed into the tissue.

Post-incubation step

At the end of the post-soak immersion each inserti was blotted on absorbent material and transferred to the 6-well plate containing 1 mL of warm assay medium. The tissues are incubated for 120 ± 15 minutes for liquid substances at standard culture conditions and 18 ± 0.25 hours for solid substances.

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MTT test

Tissue viability is assessed by MTT reduction measurement, after the post-treatment incubation.

Incubation in MTT solution

Dye solution was freshly made at the final concentration of 1 mg/mL in PBS and filtered with 0.22 µm.
24-well plates were filled with 300 µL MTT and incubated for 180 ± 10 minutes at 37°C, 5% CO_2, 95% RH.

Formazan extraction

24-well plates were filled with 2 mL of 2-propanol.
At the end of the 180 ± 10 minutes incubation in dye solution, tissues were transferred in 2-propanol and incubated for 2 hours at room temperature with gentle agitation for formazan extraction.
Plates were parafilmed to avoid solution evaporation.

Optical density measurements

At the end of the 2 hours incubation in 2-propanol tissues were pierced with a tip and the extraction solution was homogenized by pipetting up and down to complete formazan solubilisation.
2 X 200 µL aliquots per well of extraction solution were transferred in 96-well plates and optical density was read at 570 nm.

Acceptance criteria

TEST QUALITY CONTROL

The study is valid if all quality criteria are satisfied.

Negative Control acceptance criteria
Negative Control mean OD value should be > 1 e < 2.6 at 570 nm.
The difference of viability between two tissue replicates should be less than 20%.

Positive Control acceptance criteria
Positive Control has to be irritant (below 60% for 30 minute and 6 hour exposure).
The difference of viability between two tissue replicates should be less than 20%.

Test substance acceptance criteria

The difference of viability between two tissue replicates should be less than 20%.

Result calculation and data interpretation

Test substance and positive control percentage of relative viability was calculated in respect to negative control.
Tested sample was classified according to the following criteria:

Mean tissue viability ≤ 60% = IRRITANT;
Mean tissue viability > 60% = NON IRRITANT.

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RESULTS AND CONCLUSION

ACCEPTANCE CRITERIA

All acceptability criteria are satisfied.

Negative control (NC)
SOLID PROTOCOL

Mean value OD570 = 1.295 St. Dev. = 5.87%

Positive control (PC)
SOLID PROTOCOL

% mean viability = 39.24% St. Dev. = 7.21%

TEST RESULTS

TESTED PRODUCT	% CELL VIABILITY	CLASSIFICATION
	MEAN VALUE ± ST. DEV.	
DIRECTA PLUS SPA		
PURE G+	**94.28% ± 4.86%**	**NON IRRITANT**

San Martino Siccomario – 15th February 2016 (first emission)

San Martino Siccomario – 18th April 2016 (new editing)

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EVALUATION OF GENOTOXIC POTENTIAL OF A MATERIAL BY MEANS OF AMES TEST

DIRECTA PLUS SPA

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Fully paid-up share capital € 100.000 - Tax Code and VAT No. IT 03893350961
Certified Quality Management System UNI EN ISO 9001:2008 13659

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STUDY DESIGN

Title

Evaluation of the genotoxic potential of a material by means of Ames test (OECD 471)

Study aim

Aim of the test is to investigate the possible genotoxic activity exerted by the tested substance in strains of *Salmonella typhimurium,* with and without metabolic activation with S9.

Ames test allows to detect the induction of point mutations in nucleotidic bases, such as deletions, insertions, transversions and frameshift errors by using modified *Salmonella typhimurium* strains. These strains carry a defective gene in the histidine operone making them auxotrophe for this aminoacid (mutants His- which require histidine in the culture medium for growth). The method guiding principle is based on the *backmutation*: phenomenon by which bacteria exposed to a mutagenic substance may change back and become again prototrophe concerning histidine (His+), so revertant bacteria become histidine-independent. The bacterial cells, in growth phase, are exposed to different concentrations of the test agent and mutagenic activity is determined by the capacity of the test substance to induce a significant increase in the number of reverted colonies (histidine-indipendent mutant, His+) in comparison to the spontaneous reversions occurring in the control cultures.

Some chemical agents are not directly mutagen but become so following transformation and metabolic activation occurring in the organism by liver enzyme activity. In order to study this genotoxic effect, rat liver microsomial fraction (S9) has been added. S9 employ admits to identify indirect mutagen substances.

Tested product

DIRECTA PLUS SPA

PURE G+

Sample description

Powder of pristine graphene nanoplatelets

MATERIALS AND METHODS

Assay procedures - Bacterial strains

In this assay TA 1535, TA 100, TA 102, TA 1537 and TA98 strains have been used. Their characteristics are explained in Table 1.

Each tester strains contains a different type of mutation in the histidine operon. TA 1535 and TA 100 strains are specific testers for mutagens causing base substitutions. The TA 102 strain is used for detecting mutagens that require an intact excision repair system. The sensitivity of TA 100 and TA 102 is greatly enhanced by the introduction of an R factor, pKM101, which confers ampicillin resistance. Furthermore the TA 102 strain contains the multicopy plasmid, pAQ1, which confers tetracycline resistance. The frameshift tester strains used are TA 1537 and TA 98. TA 98, like TA 100, is ampicillin resistant. All *S. typhimurium* strains carry, along with the defect in the histidine gene (His-), a deep rough (rfa) character, a mutation that causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increase permeability to large molecules. At the end in these strains, except TA 102 strain, there is a delection of a gene coding for the DNA excision repair system (uvrB-), resulting in greatly increased sensitivity in detecting many mutagens. For technical reason, the delection excising the uvrB gene extends through the *bio* gene and, as a consequence, these bacteria also require biotin for growth.

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TABLE 1

S. typhimurium strain	*Affected gene*	DNA Repair Mutation	*LPS Barrier*	*Biotin Requirement*	*Plasmids*	*Mutational Events*
TA 1535	*hisG*	*uvrB-*	*rfa-*	*bio-*	-	base-pair substitution
TA 1537	*hisC*	*uvrB-*	*rfa-*	*bio-*	-	frameshift
TA 100	*hisG*	*uvrB-*	*rfa-*	*bio-*	pKM101	base-pair substitution
TA 98	*hisD*	*uvrB-*	*rfa-*	*bio-*	pKM101	frameshift
TA 102	*hisG*	*uvrB*	*rfa-*	*bio-*	pKM101 pAQ1	base-pair substitution

Test description

The study was performed using the *plate incorporation assay* with and without a 4% S9 Mix: lyophilized Aroclor 1254 rat liver induced (Moltox) supplemented with different cofactors (glucose-6-phosphate and NADP).
The experiment was performed by setting up triplicate Petri dishes containing minimum medium on which an aliquot of further medium containing histidine and biotine, the *Salmonella typhimurium* suspension, the sample to be tested at different concentrations and, if the case of metabolic activation, a 4% S9 mix were added.
Dishes have been incubated for 48 hours at 37°C. When the incubation time is over, a basal bacterial growth is achieved related to limited amount of histidine in the medium together with revertant colonies (histidine-independent).

In each Ames test assay the following parameters must be considered:

- the **negative control** (or blank) represented by dishes used to detect so called spontaneous revertant: Salmonellae that spontaneously, without any induction by the sample, revert to a normal condition.
- the **positive control** given by standard mutagen to check strains functionality

Direct test:

S. typhimurium TA 1535
S. typhimurium TA 1537
S. typhimurium TA 100
S. typhimurium TA 98
S. typhimurium TA 102

Indirect test:
All strains

Mutagens without S9

sodium azide (NaN_3)
9-aminoacridine (9AA)
sodium azide (NaN_3)
4-nitroquinoline-N-oxide (NQNO)
4-nitroquinoline-N-oxide (NQNO)

Mutagens with S9
2-aminoanthracene (2AAn)

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Mutagenesis test has been carried out with the following concentrations in dishes with and without S9:

- 5.00 mg/plate
- 1.67 mg/plate
- 0.56 mg/plate
- 0.19 mg/plate
- 0.06 mg/plate

Control dosage level

Negative control: Dimethil Sulfoxide 100µl/plate

Positive control:

STRAINS		POSITIVE CONTROLS	DOSAGE LEVELS
S. typhimurium TA 1535	Without S9	sodium azide (NaN_3) in water	1,5 µg/plate
S. typhimurium TA 1537		9-aminoacridine (9AA) in water	75 µg/plate
S. typhimurium TA 100		sodium azide (NaN_3) in water	2,0 µg/plate
S. typhimurium TA 98		4-nitroquinoline-N-oxide (4NQO) in DMSO	0,5 µg/plate
S. typhimurium TA 102		4-nitroquinoline-N-oxide (4NQO) in DMSO	1,5 µg/plate

STRAINS		POSITIVE CONTROLS	DOSAGE LEVELS
S. typhimurium TA 1535	With S9	2-aminoanthracene (2AAn) in DMSO	10 µg/plate
S. typhimurium TA 1537		2-aminoanthracene (2AAn) in DMSO	3,0 µg/plate
S. typhimurium TA 100		2-aminoanthracene (2AAn) in DMSO	3,0 µg/plate
S. typhimurium TA 98		2-aminoanthracene (2AAn) in DMSO	3,0 µg/plate
S. typhimurium TA 102		2-aminoanthracene (2AAn) in DMSO	10 µg/plate

Bacterial strains maintenance and check

The *S. typhimurium* strains were made from fresh bacterial culture derived from permanent cultures stored at -20°C in 9% DMSO. Fresh bacterial cultures were grown in Nutrient Broth (Oxoid) with ampicillin or tetracycline (for ampicillin or tetracycline resistent bacteria), were subcultivated on appropriate medium (Master plate) and stored at + 4°C for up to 2 months. The Master plates medium is particular for each strain:
the TA 1535 and TA 1537 strains grow in Minimal medium with histidine and biotine, the TA 100 and TA 98 strains grow in Minimal medium with histidine, biotine and ampicillin. The TA 102 strain grows in Minimal medium with histidine, biotine, ampicillin and tetracycline.

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The liquid culture, the permanent cultures and Master plates were checked with the following controls to confirm the tester strain genotype:

a) Check for histidine-requirement
Bacterial cultures are streaked on Minimal Medium (MM) + Bio plates and on MM + Bio + His plates. After 48h incubation at 37°C, bacterial growth should be observed on MM + Bio + His plates and shouldn't be observed on MM + Bio plates (negative control).

b) Check for the *rfa* mutation (Crystal violet sensitivity)
Culture (0,1ml) is added to 2ml of top agar and plated on Complete Medium plates. When the medium is solidified, a disc with 10µl of crystal violet solution (1 mg/ml) is put in the centre of agar surface. After 24h incubation at 37°C, a neat inhibition zone around the disc should be observed for all strains.

c) Check for the *uvrB* mutation (UV sensitivity)
Bacterial cultures are streaked on Complete Medium plates. Half plates are exposed to uvrB ray (15W germicidal lamp at 33 cm distance) for 6 seconds (TA 1535 and TA 1537) or 8 seconds (TA 100, TA 98 and TA 102). After 24h incubation at 37°C all strains should grow on the un-irradiated side of the plate only. The strain TA 102 was used as a control and should grow in all the plate.

d) Check for the *R factor* (ampicillin resistance)
Bacterial cultures are streaked on Minimal Medium + Bio + His + Amp plates. After 24h incubation at 37°C, bacterial growth should be observed for ampicillin resistance strains (TA 100, TA 98 and TA 102) only.

f) Check for the *pAQ1* plasmid (tetracycline resistance)
Bacterial cultures were streaked on Minimal Medium + Bio + His + Amp + Tet plates. After 24h incubation at 37°C, bacterial growth should be observed for tetracycline resistance strain TA 102 only.

Culture media

Liquid growth medium. This medium was prepared by dissolving 25 grams of Nutrient Broth (Oxoid) and 5 grams of NaCl in one liter of deionized water and sterilized at 1 atmosphere at 121°C for 15 minutes.

Complete medium. This medium was prepared by dissolving 25g di Nutrient Broth (Oxoid), 5g di NaCl and 15g of agar in one liter of deionized water, and it was sterilized at 1 atmosphere at 121°C for 15 minutes.

Minimal Medium (MM). The medium consisted of 15g of agar in 930ml of deionized water sterilized at 1 atmosphere at 121°C for 15 minutes. Thereafter, the temperature of the medium was brought to about 60-65°C and 50 ml glucose 40% sterile solution and 20ml of Vogel Bonner sterile solution 50X were added. About 20ml of the medium was poured into each of sterile plastic Petri plates (9 cm diameter).

The Vogel Bonner solution 50X was prepared with:

- 10g/l $MgSO_4 \cdot 7H2O$
- 100g/l citric acid · H2O
- 500 g/l K_2PO_4 anhydrous
- 175 g/l $NaNH_4HPO_4 \cdot 4H_2O$.

Top agar. This superficial medium was prepared with 6g of agar and 5g of NaCl dissolved in one litre of deionized water and sterilized at 1 atmosphere at 121°C for 15 minutes. For each 100ml of the top agar was added 10ml of 0,5mM Histidine/Biotine solution.

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Preparation of the bacterial culture.
Bacterial cell suspension were prepared by inoculating one colony of the Master culture in 25 ml liquid growth medium. The liquid culture was developed for about 16 hours at 37°C in a shaking thermostatic incubator (overnight culture).

Preparation of the S9 mix.
The S9 Mix contains a lyophilized homogenate of liver enzymes reconstituted with sterile water, prepared from adult male Sprague Dawley rats liver induced with Aroclor 1254 (Moltox). The S9 Mix was prepared immediately before use.

S9 Mix composition (50ml):
2 ml Rat liver S9 (4%)
1ml $MgCl_2$ 0.4M and KCl 1,65M
0,25 ml glucose-6-phosphato 1M
2 ml NADP 0.1M
25 ml buffer sodium phosphate 0.2M pH 7.4
19.75 ml sterile deionized water

Plate incorporation test with and without metabolic activation
The test substance or positive or negative control solutions were put into sterile test tube containing 2ml of soft top agar kept liquid in a thermostatic bath at 45°C. A suspension of Salmonella strains in stationary growth phase (0,1ml) was rapidly added. For the test with metabolic activation 0,5ml of S9 Mix was also added, instead for the test without S9 Mix 0,5ml of a physiologic solution (PBS) was added. The test tubes were shaken rapidly and the contents poured onto plates containing solid growth minimal medium. The plates were incubated at 37°C for 48 hours. Two plates per dose per Salmonella strain were prepared both for the test with and without metabolic activation. The revertant colonies per plate were counted as UFC (Colony forming units) after 48 hours incubation.

Test results evaluation
For the test to be considered valid, the following criteria must be met:

a) The sterility check must prove negative for bacterial growth.

b) the growth of all the strains must be inhibited by crystal violet; the growth of strains TA 1535 and TA 1537 must be inhibited by ampicillin, while the growth of strains TA 100, TA 98 and TA 102 must not. The growth of all strains, except TA 102, must be inhibited by tetracycline.

c) The frequency of spontaneous reversions for each strain must fall within both the range reported in the literature.

d) The activity of the S9 Mix will be confirmed by its capability to activate the positive control (which requires a metabolic transformation in order to exert its mutagenic effect).

The test substance is considered to have elicited a positive response when the number of reverted colonies increases when compared with the number of revertants in the negative controls in a dose-response related way or in a reproducible way at one or more concentrations in at least one strain with or without metabolic activation. Statistical methods may be used to decide the significance of the increase.
Any positive effect was investigate by means of specific incubation conditions in order to verify false positive possibility.

Data evaluation
The mean and the standard deviation will be calculated for reversions read in each dosage group and they will be compared with the spontaneous revertants (in the negative controls).

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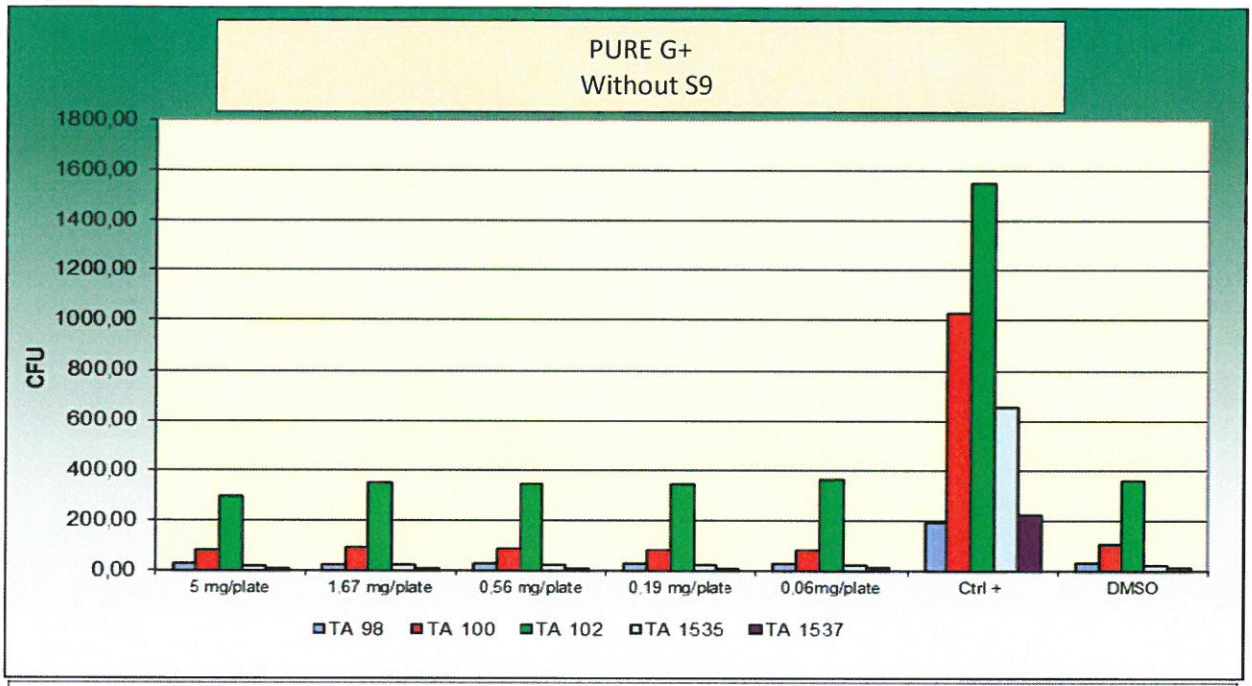
RESULTS

The following tables and graphs report the mean values as number of colonies (UFC) for revertant obtained in assay calculating 2 replicates for each dilution.

GRAPH 1



PURE G+
Without S9

mg/plate	TA1537		TA1535		TA100		TA98		TA102	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
5,00	7,7	1,15	20,0	1,00	81,7	8,14	27,0	1,00	295,7	9,07
1,67	8,3	1,53	22,0	1,00	90,0	8,72	23,3	1,53	349,3	20,13
0,56	9,3	1,53	23,3	1,15	84,0	8,00	27,3	0,58	345,7	6,03
0,19	8,3	1,15	22,0	3,46	82,7	3,21	28,7	0,58	343,0	4,58
0,06	11,7	1,53	21,7	3,79	79,7	1,53	25,3	2,52	360,7	17,62
-	-	-	-	-	-	-	-	-	-	-
Ctrl +*	223,7	4,51	654,3	47,35	1030,7	60,18	194,3	12,66	1550,0	75,50
DMSO	10,7	0,58	24,7	1,15	106,7	8,74	30,0	1,73	356,0	23,52

Ctrl +: Positive Control / DMSO: controllo negativo

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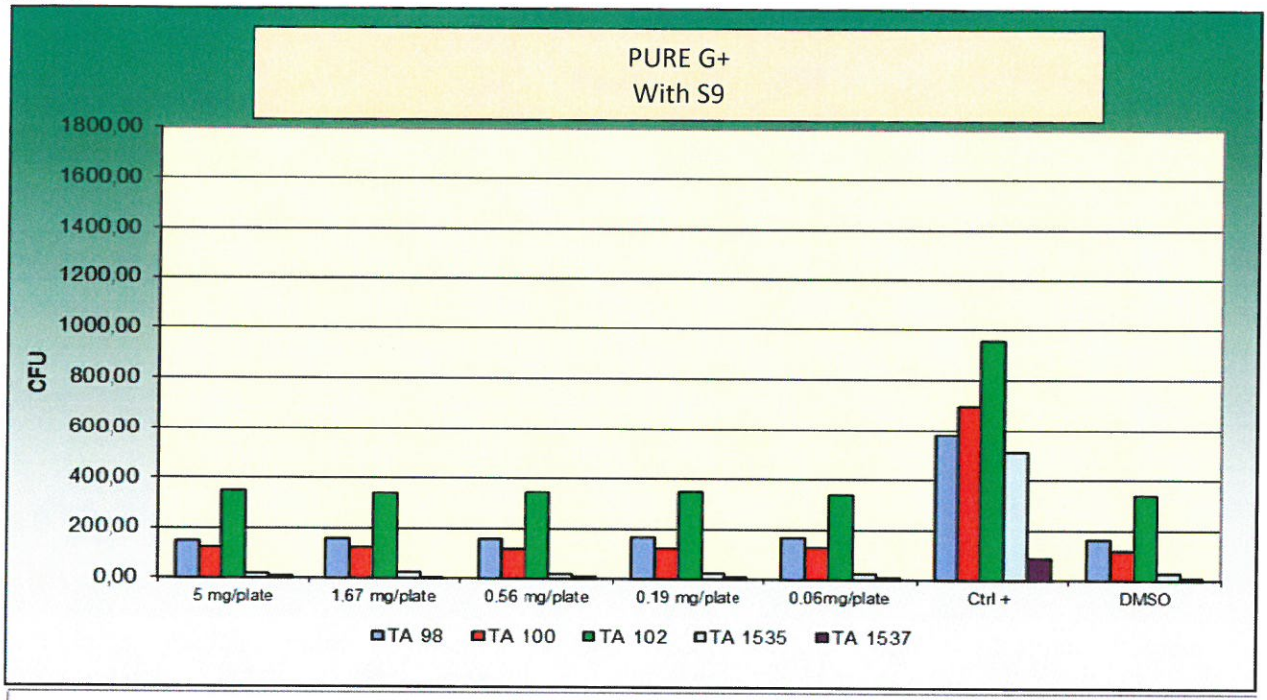
GRAPH 2



PURE G+
With S9

mg/plate	TA1537		TA1535		TA100		TA98		TA102	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
5,00	8,0	0,82	20,0	3,00	120,7	2,52	144,3	11,50	347,0	5,00
1,67	6,7	1,70	23,0	1,00	121,7	2,08	155,0	8,19	340,3	2,52
0,56	7,3	0,47	21,7	2,08	118,3	4,04	156,7	15,50	343,7	3,51
0,19	7,3	0,94	22,3	2,08	124,3	5,13	165,7	11,72	349,3	3,06
0,06	7,7	1,25	22,0	3,61	125,3	7,02	168,3	3,51	338,3	2,08
-	-	-	-	-	-	-	-	-	-	-
Ctrl +*	88,7	3,09	508,7	14,01	694,3	24,44	577,0	16,00	953,7	49,44
DMSO	10,7	1,25	29,3	2,08	119,0	1,73	163,7	14,98	336,3	10,02

Ctrl +: Positive Controls / DMSO: negative control

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CONCLUSIONS

In accordance with experimental protocol and the basis of the results from this investigation, the product

DIRECTA PLUS SPA

PURE G+

did not show any evidence of mutagenicity

San Martino Siccomario, 14 marzo 2016 (first emission)

San Martino Siccomario, 14 aprile 2016 (new editing)

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IN VITRO ANALYSIS OF THE PRO-SENSITISING POTENTIAL OF A MATERIAL

DIRECTA PLUS SPA

PURE G+

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STUDY DESIGN

Title

In vitro analysis of the pro-sensitizing potential of a material

Study aim

The aim of the test is to evaluate that the tested products do not cause pro-sensitising effects on the involved cell model.

The h-CLAT is an assay that is used to examine dendritic cell activation by quantifying changes in the expression of cell surface markers (CD86 and CD54) in the human monocyte cell line THP-1 after exposure to possible sensitizers. Expression levels of these cell surface markers are measured by flow cytometry following cell staining with fluorescently-labelled antibodies. Relative fluorescence intensity (RFI) of surface markers are compared to controls.

OECD Test Guideline is in draft, pending approval/acceptance. Previously, the h-CLAT has been evaluated in a European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) and was considered scientifically valid to be used as part of an intergrated approach to testing and assessment (IATA) for discrimination between sensitizers and non-sensitizers for hazard classification and labelling.

THP-1 cells are prototypic blood-derived immunologically active cells. On these cells we checked out the expression of two costimulatory molecules, CD54 and CD86, using a positive control as Nickel sulphate, a well known contact sensitising agents, both in in vitro and in vivo models.

When the lymphocytes T' TCR recognize the antigen (signal 1) on the Antigen-Presenting Cell (APC), additional molecules (called co-stimulatory) on the APC's membrane are necessary to obtain a complete functional immune response (signal 2).

The signal 2 is very important to define the kind of immune response that is going to be activated (umoral, cellular, etc.). The costimulatory molecules CD54 and CD86 are necessary to obtain an efficient antigen presentation by the T cell receptor (TCR) and hence to obtain a correct immune response. CD54 is a membrane glycoprotein expressed on the surface of different antigen-presenting cells (dendritic cells, Langerhans cells, monocytes/macrophages, keratinocytes); CD86 is a membrane protein of the immunoglobulin superfamily, with costimulatory function too. Both CD54 and CD86 recognise a further molecule, a glycoprotein called CD28 on the T lymphocyte membrane.

The switching on of the ligand/receptor system CD28/B7 avoids the T cell apoptosis and sustains their proliferation and differentiation and the production of many cytokines.

The increasing level of expression of CD54 and CD86 on monocytes is a signal of activation of the immune response derived from the exposition to a potentially sensitising antigen.

Tested sample

DIRECTA PLUS SPA

PURE G+

Sample description

Powder of pristine graphene nanoplatelets

Evalued parameter and experimental model

The experimental study provided the execution of the following determinations:

- Preliminary evaluation of cytotoxicity on human monocytes through MTT assay
- Evaluation of pro-sensitising activity on human monocytes through FACS analysis

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The test was carried out on a monocyte-like human line called THP-1. Cells are kept in RPMI containing 10% FCS and 2 mM glutamine.

Test references

The test was carried out using a positive control:

- Nickel Sul fate cristals dissolved in PBS

EXPERIMENTAL PROTOCOL

Sample preparation

The sample was eluted in culture medium for 24 hours at 37°C with ratio 10 mg/ml. The eluted was diluted in the cell culture medium at different concentrations for the preliminary cytotoxicity screening on the THP-1 cells aimed to decide the best concentration to test it without cytotoxic effects on the cells, in order to avoid false results. Cell medium exposed to the same experimental conditions was used as a negative control.

Analysis of cytotoxic potential (MTT)

The MTT assay is simple, accurate and yields reproducible results. This method has been developed originally by Mossman. The key component is (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) or MTT. This product is of yellowish colour in solution. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, leading to the formation of purple crystals which are insoluble in aqueous solutions. The crystals are re-dissolved in acidified isopropanol and the resulting purple solution is measured spectrophotometrically. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material.
MTT-medium is prepared as described. After exposure of the cells to the test material, the cells are washed with PBS and exposed to the MTT-medium at 37°C.
At the end of the incubation period, the MTT-medium is removed and the cells receive the MTT solubilization solution. The plate is shaken on a rotatory plate shaker for 20-30 minutes, ensuring that all the crystals have dissolved from the cells and have formed a homogeneous solution.
The absorbance is measured as described with background elimination.
The results are expressed in terms of viability:

% cell viability = (OD treated cells/ OD untreated cells) x 100

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Analysis of pro-sensitizing potential

After the incubation with the tested substance and the controls, cells are collected, checked under the microscope for their vitality by staining with Trypan Blue dye and counting in a cell counter chamber, washed in PBS and then marked with a fluoresceinated anti-CD54 or CD86 antibody. After washing, to eliminate the excess antibody, the MFI (Mean Fluorescence Intensity) linked to the cells was evaluated by means of a flux cytofluorimeter (FACS, Fluorescence Activated Cell Sorter, Becton Dickinson, Mountain View, CA). This value is proportional to the expression of costimulatory molecules.

Positive control are THP-1 culture cells exposed to Nickel Sulfate; the concentrations of two pro-sensitizing agents used as reference substances are chosen among thatrisulted "not cytotoxic" in the preliminary MTT test. The MFI of the non-treated THP-1 cells and of cells after reaction with a monoclonal isotype-matched antibody was used as an internal control (basal fluorescence).

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RESULTS

In table 1 are reported the results expressed as co-stimulatory molecules expression after the exposition of the monocyte cell line THP-1 for 24 hours to the investigated sample and control substances. The data significantly different by negative control (CTR-) are highlighted with * (T-test, $p<0.05$).

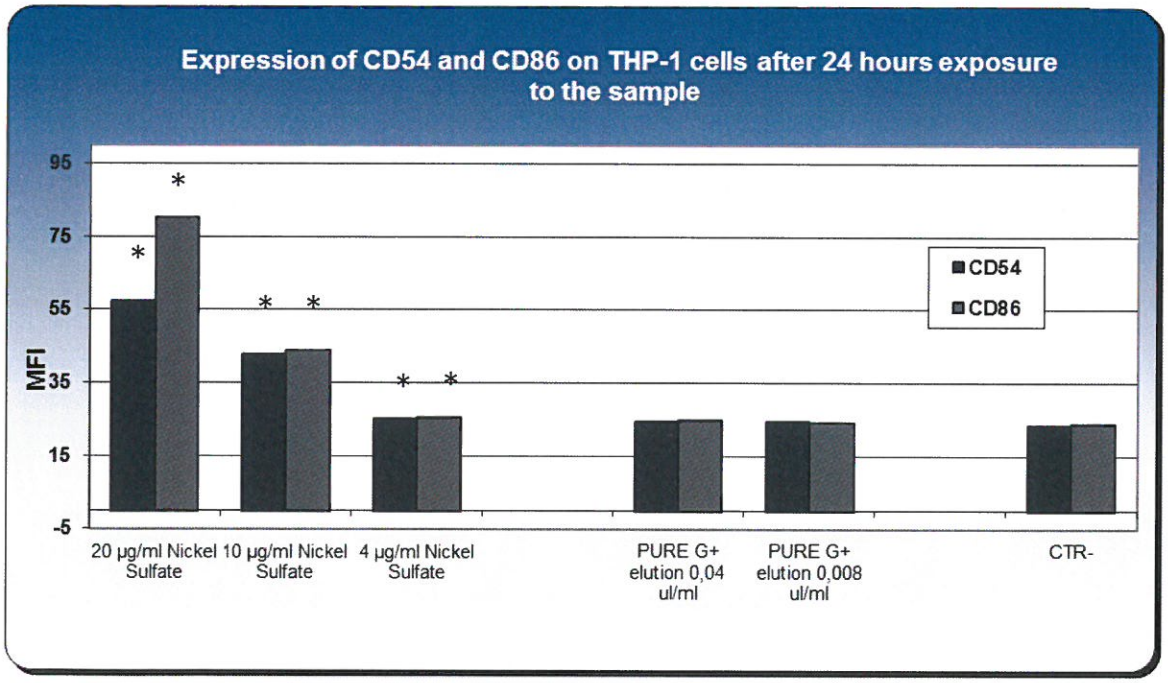
TABLE 1

Samples	CD54 (MFI*)	CD86 (MFI*)
20 µg/ml Nickel Sulfate	57,25*	80,35*
10 µg/ml Nickel Sulfate	42,73*	43,99*
4 µg/ml Nickel Sulfate	25,04*	25,44*
CTR-	23,55	23,67
PURE G+ elution 0,04 ul/ml	24,50	24,89
PURE G+ elution 0,008 ul/ml	24,52	24,15

*MFI = Mean Fluorescence Intensity - it is the geometric average of the fluorescence intensity of the cells decorated with the fluoresceinated antibody and it is proportional t the number of stained molecules per cell.

FIGURE 1

In figure 1 the values of expression of CD54 and CD86 found for the tested products and the relative contı



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CONCLUSIONS

The evaluated substance does not affect the expression of both the evalued markers in immunocompetent cells in vitro and hence it does not show a stimulating potential on the immune response mediated by monocyte/macrophage.

We can hereby state that the product named:

DIRECTA PLUS SPA

PURE G+

does not show any detectable pro-sensitising effect

San Martino Siccomario – 29th February 2016 (first emission)

San Martino Siccomario – 19th April 2016 (new editing)

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ANALYSIS OF THE REPRODUCTIVE TOXIC POTENTIAL OF A MATERIAL

DIRECTA PLUS SPA

PURE G+

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STUDY DESIGN

Title

Evaluation of the reproductive toxic potential of a material by means of in vitro maturation test on bovine oocytes (bIVM test)

Study aim

The aim of the study is to evaluate the in vitro reproductive toxicity of products and mixtures on the basis of the specific toxicity on bovine oocytes during the process of in vitro maturation applying the bovine oocyte in vitro maturation test (bIVM test, Invittox protocol 129). This test consists in the exposure of immature bovine oocytes to test item during the process of in vitro oocyte maturation (Fig. 1). During this process the oocytes resume meiosis and achieve the stage of metaphase II that corresponds to the stage suitable for fertilisation and subsequent embryonic development. The purpose of the oocyte maturation test is to monitor the substance effects on the oocytes with special reference to the nuclear configuration changes within the oocyte as compared to control non-exposed oocytes. The process of oocytes in vitro maturation mimics the in vivo process of ovulation, that is a very sensitive and crucial step of the reproductive cycle.

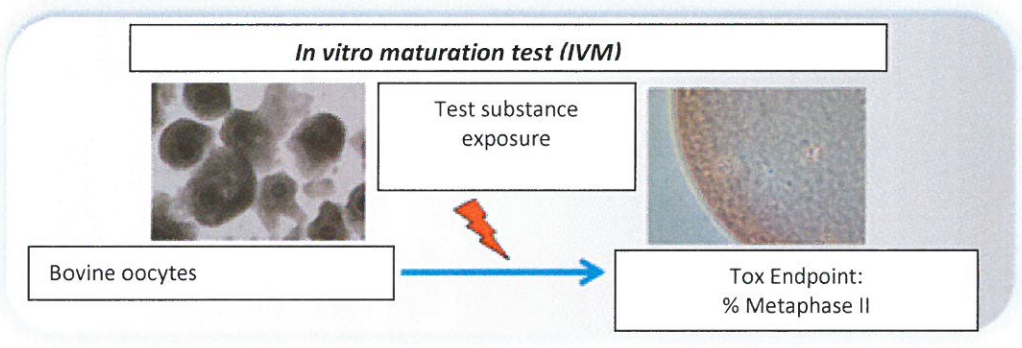

Fig. 1. Bovine oocytes in vitro maturation test (bIVM).

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Tested sample

DIRECTA PLUS SPA

PURE G+

Powder of pristine graphene nanoplatelets

Evaluated parameters and experimental model

The toxicological endpoint evaluated for the bovine oocyte in vitro maturation test is the completion of meiosis up to metaphase II stage, which is reached after 24h of in vitro culture.
The test was carried out on bovine cumulus/oocyte complexes (COCs), assay functional unit. Each COC is composed of the bovine oocyte surrounded by the closely associated follicular cells, more precisely called cumulus cells.
If possible, EC_{50} value (50% inhibition of maturation process) is obtained. This value is compared to EC_{50} 50 µM cut-off: EC_{50} value lower than 50 µM are identified as potentially toxic (prediction model).

Test references

The test was carried out using a positive control:

- Cycloheximide 0.39 µM

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EXPERIMENTAL PROTOCOL

Test system procurement and maintenance

Ovaries were recovered at the abattoir from pubertal females subjected to routine veterinary inspection and in accordance to the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications. The ovaries were placed in an insulated container in sterile PBS and transferred, in a warm box kept at 25°C, to the laboratory. Immature bovine oocytes, surrounded by the cumulus/follicular cells, were recovered from ovarian follicles by dissection or aspiration (Fig. 2).

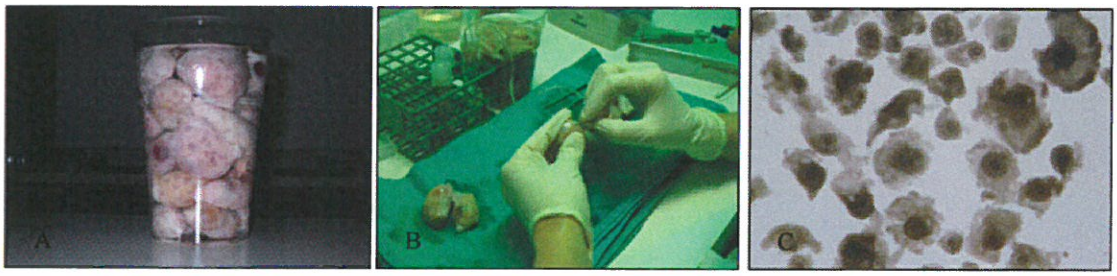

Fig. 2.A: bovine ovaries; B: aspiration of ovarian follicles; C: bovine oocytes surrounded by cumulus cell.

Sample preparation

Test substance was dissolved in tissue culture medium for oocyte maturation TCM199 (Sigma cat. n° M2154) at concentration of 50 mg/ml. The suspension was mechanically stirred, left in incubation at 37°C for 16h and then centrifuged (30 minutes at 1500g) twice. The surnatant was collected and filtered through a 0.22 micron sterile filter (the first 2 ml of the filtrated was discarded to condition the filter) and was labelled as stock solutions. At the end of the incubation the stock solution appeared colourless and the pH was 8.0-8.5. The osmolarity of the solution was also measured and the range of values was 300-308 mOsm.

The eluate was diluted in culture medium and three different concentrations were used for the assay. The oocytes were incubated in a sterile plastic tube or in a 0.5 ml well of a 4-wells plate, in a 5% CO_2 incubator in saturated humidity for 24h at 38.5°C.

Test material exposure procedures

Oocyte maturation

- Immature oocytes were recovered from follicles with a diameter of ≥ 3mm by dissection or aspiration in tissue culture medium for oocyte collection (TCM 199, Sigma cat. n° M2154, supplemented with: 25 mM Hepes, 1 mg/ml PVA, 0.10 mg/ml glutamine, 10 µg/ml heparin). The oocytes were selected on the basis of their homogenous cytoplasm surrounded by compact cumulus cells (Fig. 3A).
- Following two washes in 2 ml of collection medium the oocytes were incubated in tissue culture medium for oocyte maturation (TCM 199 , Sigma cat. n° M2154, supplemented with: 1mg/ml of polyvinil alcohol, 10 ng/ml Epidermal Growth Factor, 0.05 IU of each FSH/LH, 0.10 mg/ml glutamine, 0.11 mg/ml sodium pyruvate) in a

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sterile plastic tube or in a 0.5 ml well of a 4-wells plate (containing max 30 oocytes per 0.5 ml) , in a 5% CO_2 incubator in saturated humidity for 24h at 38.5°C. At the end of maturation the morphology of the cumulus

oocytes complexes changes considerably due to the expansion of the cumulus cells that appear as a loose cloud of cells surrounding the oocytes (Fig. 3B).

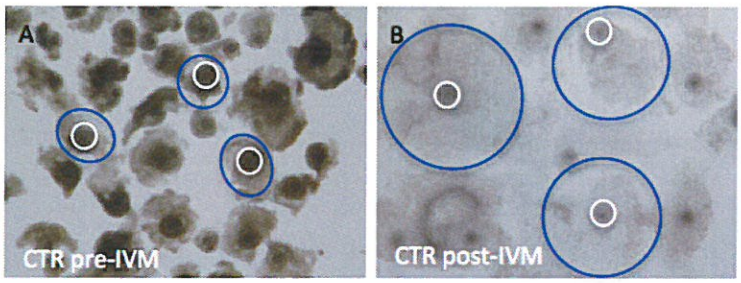

Fig. 3A: bovine oocytes surrounded by a compact cumulus immediately after recovery form bovine ovaries; 3B: bovine oocytes surrounded by an expanded cumulus and follicular cells after 24h of in vitro maturation. The white circles indicate, as examples, 3 oocytes and the blu circles indicate the cumulus cells. After maturation the cumulus cells appear very expanded due to the production of extracellular matrix by the cells themselves.

Chromatin staining

- At 24h from the beginning of incubation the oocytes were removed from the maturation medium, denuded of cumulus cells using a fire pulled glass pasteur pipette, placed on a slide in small drops of about 0.5 µl and fixed in acetic acid/ethanol solution for 18-24h.
- Finally the slides were stained with lacmoid by allowing the staining solution to enter by capillarity between the slide and the cover slip in order to stain the oocytes.
- The slides were then examined to evaluate the oocyte nuclear morphology by microscopic observation at 200-400x magnification in phase contrast.

Endpoint measurement

The toxicological endpoint evaluated for the bovine oocyte in vitro maturation test is the completion of meiosis up to the metaphase II stage, which is reached after 24h of in vitro culture (Fig. 4). At that time the oocytes are fixed (ethanol/acetic acid) on a slide and stained (Lacmoid) to analyse the chromatin configuration. The slides are then examined to evaluate the oocyte nuclear morphology by microscopic observation at 200-400x magnification in phase contrast. Matured oocytes are characterised by the extrusion of the first polar body associated to the second metaphase plate. Not matured oocytes are arrested at one of the following meiotic stages: germinal vescicle stage (GV), metaphase I, anaphase I. Abnormal metaphase plates are recorded as disorganised metaphase plates.

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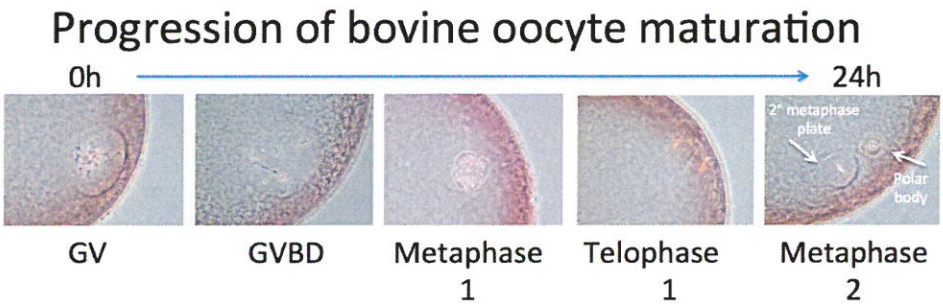

Fig. 4: Cytological images of oocytes fixed and stained at different time after the start of maturation. The metaphase 2 stage is reached after 22-24h of in vitro maturation.

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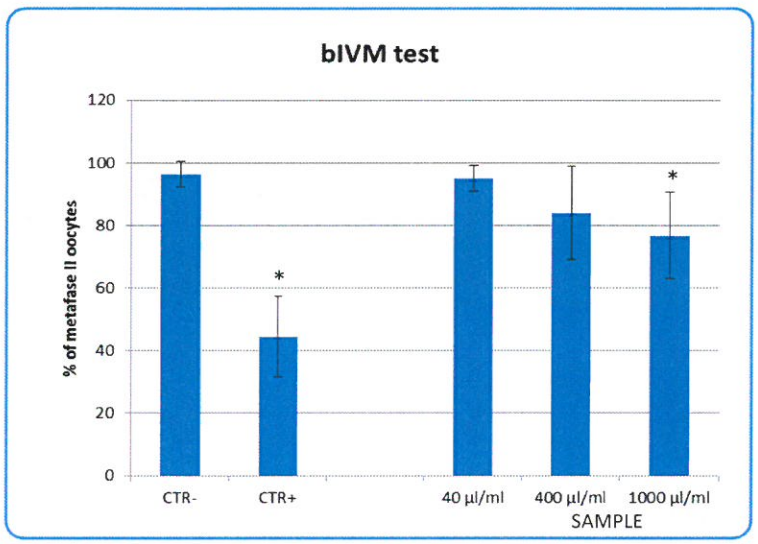
RESULTS

Below are reported the results for the sample tested at different experimental concentrations (% of metaphase II oocytes). Results indicate that the exposure to test substance does not affect the expansion of the cumulus cells and the morphology of the cumulus-oocytes complexes (Fig. 6). However a minor inhibition on the maturation process is observed only at the maximum tested concentration (Fig. 5).



		% Met. II	Dev. St.
CTR-		96,36	4,12
CTR+		44,44	12,96
PURE G+	40 µl/ml	95,12	4,12
PURE G+	400 µl/ml	83,93	14,91
PURE G+	1000 µl/ml	76,79	13,81

Fig. 5: Results of the bIVM test. Asterisks indicate statistical difference versus negative control (T-Student test, p<0,05*).

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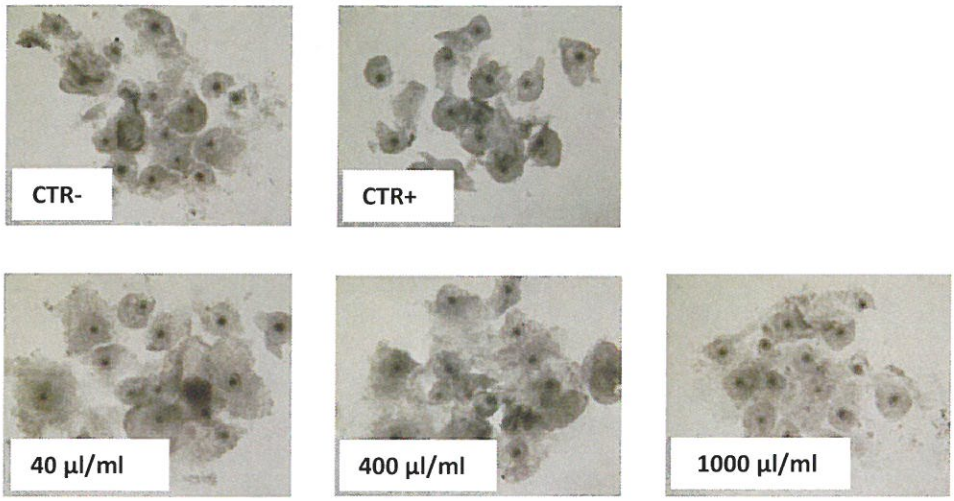

Fig. 6: Results of the bIVM test. Representative images of the different treatments (stereomicroscope, bright field, magnification 12,5x).

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CONCLUSION

The adopted prediction model states that for the maturation endpoint chemicals with an EC_{50} value lower than 50 uM are identified as affecting the maturation process (positive); in contrast, for compounds with an EC_{50} value higher than 50 µM the prediction model classifies the substances as negative (Invittox protocol n°129).
Considering the test substance composition and chemical-physical properties provided by the Customer estimated molar concentrations tested are 4.17 mM (1000 µl/ml), 1.67 mM (400 µl/ml) and 167 µM (40 µl/ml).
Only the stock solution (1000 µl/ml) revealed a toxic effect on oocyte maturation, while all other concentrations did not show a reproductive toxicity potential.
Based on the obtained results all non-toxic tested concentrations are higher than the limit value of 50 µM, threshold under which the bIVM test detects reproductive toxicity.

We can hereby state that the product named:

DIRECTA PLUS SPA

PURE G+

doesn't affect the in vitro maturation process of bovine oocytes

San Martino Siccomario - 21st March 2016 (first emission)

San Martino Siccomario – 19th April 2016 (new editing)

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IN VITRO PRODUCT SAFETY STUDY
BIOLOGICAL EVALUATION OF A MATERIAL
IN VITRO SKIN CORROSION: HUMAN SKIN MODEL TEST FOR THE EVALUATION OF SKIN CORROSION

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TEST PROCEDURE INFORMATION

Tested product

DIRECTA PLUS SPA

PURE G+

Sample description
Powder of pristine graphene nanoplatelets

Date of test execution
23/12/2015

Experimental model
STERLAB EPIDERMIS age 17 batch N° 1512 EPID 01

0,5 cm^2 reconstructed epidermis of EP.AJ P1 21. keratinocytes. Cells are grown on inert polycarbonate filter on chemically defined medium, airlifted for 17 days.

Culture medium
MAINTENANCE MEDIUM batch N° 1512 HC 629

Protocol
In vitro Skin Corrosion: Human Skin Model Test (OECD 431)

Positive control
Glacial acetic acid

Negative control
Deionized water

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SUMMARY

The purpose of the study is the evaluation of skin corrosivity of a material by using the alternative method «In vitro Skin Corrosion: Human Skin Model Test» (OECD 431). The salient features of this method are reported below:

- study performed on reconstituted human epidermis STERLAB EPIDERMIS, a three-dimensional human skin model with a functional stratum corneum;
- deposit of 20 mg ± 3 mg of test material on the surface of epidermis for 3 and 60 minutes;
- assessment of cell viability by MTT method. Validation of the test by positive control (Glacial acetic acid) and negative control (deionized water).

In accordo con i risultati della vitalità cellulare misurata dopo il trattamento con la sostanza in esame (rispettivamente 99.13% dopo 3 minuti di applicazione e 92.85% dopo 60 minuti di applicazione), il prodotto

In accordance with the results of cell viability obtained after tissue treatment (respectively 99.13% after 3 minutes application and 92.85% after 60 minutes application) the product

DIRECTA PLUS SPA

PURE G+

is classified as NON CORROSIVE

INTRODUCTION

Study aim

The «In vitro Skin Corrosion: Human Skin Model Test » (OECD 431) is designed for the prediction of skin corrosivity of chemicals by measurement of ability to produce a decrease in cell viability, as reflected in the MTT assay, on the reconstructed human epidermis model. The principle of the human skin model assay is based on the hypothesis that corrosive chemicals are able to penetrate the stratum corneum by diffusion or erosion and are cytotoxic to the underlying cell layers.

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Bibliographic references

This test takes in consideration the following bibliographic references:

SKINETHIC SKIN CORROSION TEST (SOP): Validation of the Skinethic skin corrosion assay for the prediction of skin corrosion of chemicals using the 0,5 cm^2 RHE model, 2009;

OECD Guidelines for the testing of chemicals, OECD 431: In Vitro Skin Corrosion: Human Skin Model Test Epidermis Test Method, 2004;

INVITTOX protocol n. 118/119 (EpiSkinTM Skin Corrosivity Test/ EpiDermTM Skin Corrosivity Test).

Tested product

DIRECTA PLUS SPA

PURE G+

Sample description

Powder of pristine graphene nanoplatelets

MATERIALS AND METHOD

Sample and control preparation

Liquid and viscous test substances:

40 µL ± 0.5 µL of the undiluted test substance.

Solid test substances:

20 mg ± 3 mg of undiluted test substance.

Two tissues per test substance are used.

Control preparation

Glacial acetic acid was used as positive control. Deionized water was used as negative control.

Tissues

RHE set and media are provided by STERLAB.

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Together with the tissues are also provided material quality control that are satisfied: normal histology (absence of significative alterations), cell viability (MTT OD > 0.8), barrier function integrity (4.00 < ET50 < 9.00 hrs), absence of bacteria, fungi, HIV, and Hepatitis B, C.

Procedure for cell viability evaluation

Pre-incubation step

An appropriate number of 24-well plates was filled with 300 µL maintenance culture medium.
Using sterile forceps, tissues were taken off from agarose and the bottom of the insert wass cleaned on sterile absorbent paper to remove eventual remaining agarose pieces.
The tissues were transferred on fresh medium and placed at 37°C, 5% CO_2, 95% RH incubator until test substance application.

Application of test substance and rinsing

24-well plates were filled with 300 µL maintenance culture medium pre-warmed at room temperature:
Using sterile forceps, tissues were transferred in the new plates.
Test was performed in double: 2 wells per test substance, 2 for positive control and 2 for negative control for each time point.

Topical application: 3 minutes and 60 minutes treatment

Liquid and viscous test substances:

40 µL ± 0.5 µL (i.e. 80 mg/cm²) of the undiluted test substance are applied on the top of each epidermis tissue using micropipette.

Solid test substances:

20 mg ± 3 mg (i.e. 40 mg/cm²) of the undiluted substance are applied to the epidermis surface; 20 µL of deionized water are added to ensure a good contact of the sample with the skin surface.

Rinsing and drying steps

Tissues were rinsed with 25 mL PBS to remove all residual test substance from the epidermal surface. Insert bottom was dried on a sterile absorbent paper. The surface of the tissues was dried with sterile cotton tip.

MTT test

Tissue viability is assessed by MTT reduction measurement, after the 3 minutes and 60 minutes treatments.

Incubation in MTT solution

Dye solution was freshly made at the final concentration of 1 mg/ml in PBS and filtered with 0.22 µm.
24-well plates were filled with 300 µL MTT and incubated for 3 hours (± 5 minutes) at 37°C, 5% CO_2, 95% RH.

Formazan extraction

24-well plates were filled with 800 µL 2-propanol.
At the end of the 3 hours (± 5 minutes) incubation in dye solution, tissues were transferred in 2-propanol.
700 µL 2-propanol were added on the top of each tissue and incubated for 2 hours (± 5 minutes) at room temperature with gentle agitation for formazan extraction.
Plates were parafilmed to avoid solution evaporation.

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Optical density measurements

At the end of the 2 hours (± 5 minutes) incubation in 2-propanol tissues were pierced with a tip and the extraction solution was homogenized by pipetting up and down to complete formazan solubilization.
3 X 200 µL aliquots per well of extraction solution were transferred in 96-well plates and optical density was read at 570 nm.

Acceptance criteria

TEST QUALITY CONTROL
The study is valid if all quality criteria are satisfied.

Negative Control acceptance criteria
Negative Control mean OD value should be > 0.8 at 570 nm.
Vitality coefficient of variation value should be < 30% (Performance Standards ECVAM SIVS, 2007).

Positive Control acceptance criteria
Positive Control has to be corrosive.
Vitality coefficient of variation value should be < 30% (Performance Standards ECVAM SIVS, 2007).

Test substance acceptance criteria

Vitality coefficient of variation should be < 30% (Performance Standards ECVAM SIVS, 2007).

Result calculation and data interpretation

Test substance and positive control percentage of relative viability was calculated in respect to negative control.
Tested sample was classified according to the following criteria:

Mean tissue viability < 50% after 3 minutes or ≥ 50% after 3 minutes and < 15% after 1 hour = CORROSIVE;
Mean tissue viability ≥ 50% after 3 minutes and ≥ 15 % after 1 hour = NON CORROSIVE.

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RESULTS AND CONCLUSION

ACCEPTANCE CRITERIA

All acceptability criteria are satisfied.

Negative control (NC)		
3 MINUTES	Mean value OD570 = 1.387	St. Dev. = 1.48%
1 HOUR	Mean value OD570 = 1.244	St. Dev. = 1.27%
Positive control (PC)		
3 MINUTES	% mean viability = 5.91%	St. Dev. = 0.61%
1 HOUR	% mean viability = 4.18%	St. Dev. = 0.45%

TEST RESULTS

TESTED PRODUCT	% CELL VIABILITY MEAN VALUE ± ST. DEV.	CLASSIFICATION
DIRECTA PLUS SPA PURE G+	**99.13% ± 1.68%** after 3 minutes application **92.85% ± 2.05%** after 60 minutes application	NON CORROSIVE

San Martino Siccomario – 21st January 2016 (first emission)

San Martino Siccomario – 14th April 2016 (new editing)

Experimenter

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COMPLIFE GROUP

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Sistema di Gestione Qualità Certificato UNI EN ISO 9001:2008 13659

UNI EN ISO 9001:2008 CERTIQUALITY SISTEMA DI GESTIONE QUALITÀ CERTIFICATO
CERTIQUALITY È MEMBRO DELLA FEDERAZIONE CISQ IQNet

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FINAL REPORT **B-02523**

Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

05th June 2018

Test facility
VIVOTECNIA Research
C/Santiago Grisolía, 2
28760 Tres Cantos
Madrid, Spain

CONTENTS

1 ORGANISATION AND RESPONSIBLITIES

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2 ADDITIONAL RELEVANT PERSONNEL INVOLVED IN THE STUDY

Name	Initials	Position
Raquel Gómez	RGR	Animal facility director and Welfare adviser
Verónica Maillo	VMA	Veterinarian
Jesús Illán	JIM	Technician responsible
Sergio Prieto	SPC	Archivist

3 DECLARATIONS AND SIGNATURES

Study director

I hereby certify that the present study was conducted according to the Good Laboratory Practice (GLP) requirements of European Directive 2004/10/EC and Spanish law RD 1369/2000.

The work was conducted according to the study plan.

- All applicable SOPs have been followed.
- Raw data have been recorded accurately.
- Deviations to the study plan have been documented at the test facility, and acknowledged by the study director.

05.06.2018

Dr. Pilar Prieto
Date and signature

Laboratory director

I hereby certify that a sufficient number of qualified personnel, appropriate facilities, equipment and materials were available to ensure that the study was conducted in an appropriate and timely manner.

Dr. Andrés Konig 05.06.18
Date and signature

Quality assurance

The following inspections and audits have been carried out by Vivotecnia's quality assurance unit (QAU) in relation to this study:

Study Phase	Date(s) of inspections	Date of reporting to study director & laboratory director
Study plan Specific Supplement audit	24.04.2018	24.04.2018
Aerosol generation; exposure; test item usage; animal housing and clinical observations	25.04.2018	26.04.2018
Raw data	01.06.2018	01.06.2018
Final report audit	01.06.2018	01.06.2018

Process and facility based inspections: Inspections of procedures and facilities where this study was conducted were carried out. These were conducted and reported to appropriate Vivotecnia Management as indicated bellow.

Process / Facility based inspections	Date(s) of inspections	Date of reporting to technical director & laboratory director
Facilities: rodent animal facility; big animals animal facility	14.02.18	14.02.18
Aerosol generation and animal exposure process	25.04.18	26.04.18
TPM and PSD measurement process	20.03.18	21.03.18
Clinical observations inhalation process	20.03.18	21.03.18
Facilities: inhalation unit	18.05.17	22.05.17
Archiving process and facilities	03.04.18	06.04.18
Inhalation process (personnel and type of product)	20.03.18	21.03.18

This statement also confirms that this final report accurately and completely reflects the raw data.

05.06.18

María Herrero
Date and signature

3.1 ADHERENCE TO PROTOCOL

3.1.1 DEVIATIONS TO THE STUDY PLAN

- Deviations to the study plan were documented at the test facility and were acknowledged by the study director. Any deviation was reported to the study monitor as soon as possible and is documented in present final report.
- The study director assessed the impact of the deviations on the quality and / or integrity of the study.

4 STUDY CALENDAR

The study was conducted according to the following calendar:

Milestone	Date
Study initiation	24.04.2018
Experimental starting	25.04.2018
Experimental completion	09.05.2018
Draft report issue	22.05.2018
Study finalization	05.06.2018

5 SUMMARY

Introduction and experimental design

The present study has been designed to evaluate the acute inhalation toxicity of the test item PURE G + in male and female Sprague Dawley rats by the traditional protocol described in the OECD Guideline Nº 403: Acute Inhalation Toxicity. This method provides lethality data of the test item and allows its classification according to the Globally Harmonized System (GHS) of Classification and Labelling of Chemicals.

For that purpose, a group of three male and three female Sprague Dawley rats was exposed by nose-only, flow-past inhalation to PURE G+ at a mean concentration of 1.87 mg/L air during 4 hours. This concentration was found to be the highest technically achievable.

All animals were observed for clinical signs and mortality during the exposure and the subsequent 14-day observation period. Body weight was recorded just before starting exposure, 24 h, 72 h and one week thereafter and also before sacrifice two weeks after exposure.

At the end of the observation period, all surviving animals were subjected to a gross necropsy and all macroscopic abnormalities were recorded.

Results and Conclusions

The ranges of aerosol concentration, temperature, relative humidity and air flow rate were considered satisfactory for a study of this type. In addition, the test item was considered to be respirable to rats.

One male animal was found dead the day after exposure (study day 2). Upon gross necropsy, black spots were observed in the lungs and larynx, trachea, oesophagus and stomach were black colored. Furthermore, black contents in the stomach were also present. Immediately after exposure, this animal presented dirty head fur (blackish), prostration, vocalization and breathing difficulty, signs that remained one hour after finishing exposure.

Apart from dirty fur on the head area, piloerection, prostration and breathing difficulty were observed in the other two males 1 hour after finishing exposure but these signs were not present the day after.

In the three females, black fur on the head was also observed until study day 8. Additionally, transient piloerection was observed in one animal only 1 hour after finishing exposure.

From study day 9 until the end of the 14 day-observation period, no clinical signs were observed in any of the five surviving animals and all of them exhibited a normal behavior.

A decrease in mean body weight of approximately 11% in males and 3% in females was observed between study day 1 (exposure) and study day 2. Thereafter body weight tended to increase gradually in most animals and mean body weight gains over the 14 day observation period of 19% and 10% were recorded for males and females, respectively.

In all the surviving animals (two males and three females) black spots were observed in the lungs upon gross necropsy. The lungs were preserved in neutral-buffered 4 % formaldehyde.

It can be concluded that, under the present experimental conditions:

- The exposure of male and female Sprague Dawley rats to the test item PURE G + for 4 hours resulted in the premature death of one male animal.
- The respiratory effects of the test item were more evident in males than in females.
- The marginal body weight loss between study days 1 and 2 in all animals was considered to be mainly due to the stress related to the nose-only exposure procedure, although a contribution of the treatment with the test item cannot be excluded.
- The presence black spots in the lungs of all animals was considered to be related to test item exposure.
- The LC_{50} of PURE G + was greater than 1.87 mg/L air (gravimetric aerosol concentration).
- Based on the GHS classification criteria, PURE G + can be considered to be under human health hazard Category 4 (“harmful if inhaled”) or higher.

6 OBJECTIVE

The present study has been designed to evaluate the acute inhalation toxicity of the test item PURE G + in male and female Sprague Dawley rats by the traditional protocol described in the OECD Guideline Nº 403: Acute Inhalation Toxicity. This method provides lethality data of the test item and allows its classification according to the Globally Harmonized System (GHS) of Classification and Labelling of Chemicals.

7 TEST SYSTEM AND CONDITIONS

7.1 TEST SYSTEM CHARACTERISATION

Species:	Rat
Strain:	Hsd: Sprague Dawley® SD®
Sex:	Male / Female
Colour:	*Albino*
Supplier:	Envigo RMS Spain S.L. Ctra. St. Miquel del Fai, Km. 3.5 08182 - Sant Feliú de Codines Barcelona – Spain
Health status*:	Specific Pathogen Free (SPF). Females will be nulliparous and non-pregnant
Rationale for selection of species / strain:	The Sprague Dawley rat is a suitable rodent strain, acceptable to regulatory authorities as a recommended rodent test system, and for which extensive background data are available
Total number of animals:	Limit test: 6 animals in a single group (3 males and 3 females)
Age of the animals at exposure:	8 weeks
Animals per cage:	3 of the same sex
Bedding material:	Sodispan (SR-CHOPO-T) (autoclavable)
Change of cages:	At least once a week and when deemed necessary throughout the study period
Inclusion criteria on arrival:	Veterinary inspection
Acclimatisation period:	Animals were acclimatised to Vivotecnia housing facilities 7 days prior exposure. Acclimatisation to the nose-only restraining tubes was performed for at least 30 minutes the day of exposure.
Animal identification:	Digit ink marks

**The health monitoring report provided by the animal supplier will be stored at Vivotecnia.*

7.2 ANIMAL FACILITIES

Location:	Vivotecnia Research animal facilities C/Santiago Grisolía, 2 28760 Tres Cantos (Madrid) Spain
Accreditation authority:	Dirección General de Agricultura, Comunidad de Madrid (ES 289030000025)
Applicable law:	European Directives 2010/63/UE and Spanish Law RD 53/2013
Target parameters*	
Light cycle:	12:12 – 07.00 to 19.00 CET
Temperature:	19.8 – 25.6º C
Relative humidity:	27 – 49%

**Environmental parameters will be classified, evaluated, recorded and reported on a daily basis according to the internal SOPs.*

7.3 ANIMAL WELFARE

Whenever possible animals were kept in groups. Enrichment devices (nesting material, tubes, and chew blocks) were provided as a default husbandry practice.

7.4 ANIMAL DIET

Food:	Teklad global diet
Reference:	2914C
Supplier:	Envigo Station Road, Blackthorn, Bicester Oxon, OX25 1TP United Kingdom
Nutritional / contaminant contents:	Certificate of analysis for the batch used in the study is included in Annex III
Food availability:	*Ad libitum* (animals were deprived of food during exposure)
Drinking water:	Tap water
Watering:	Bottles
Quality control*:	Certificate of analysis is included in Annex III
Water availability:	*Ad libitum* (animals were deprived of water during exposure)

**Contaminants in the diet / drinking water are considered not to be present at levels at which they might interfere with the quality and objectives of the study.*

8 TEST ITEM IDENTIFICATION

8.1 TEST ITEM IDENTIFICATION

Test item: PURE G+	
Batch:	P219/2016
Supplier:	Directa Plus S.p.A
Identification code:	Lab4LIFE ID: 180226-01
Physical and chemical properties	
Physical state:	Solid: Powder
Purity / composition:	> 98%
Correction factor:	None
Colour:	Black-grey
Storage conditions:	Room temperature (ca. 23º C) and protected from light
Homogeneity:	Neat substance
Expiry / retest date:	Not defined*
Handling conditions:	During manipulation of material under chemical hood, set the front glass and the fan speed so that the frontal suction hood is between 0.4 and 0.7 m/s. The height of the frontal safety glass must be kept at 30 cm from the bottom, while maintaining the proper frontal speed. Manipulate the material in order to ensure the least possible dispersion of material. Avoid or minimize the presence of air currents near the chemical hood. Provide a 5 minutes break at the end of the nanoparticle manipulation before proceeding with other activities to allow the concentration of aerodispersed particles to be reduced in the hood. During the activity, use personal protective equipment for handling the material (FFP3 mask, gloves). Proceed to cleaning the hood workpiece after any nanomaterial manipulation activity: the damp cloth is sufficiently efficient; equivalent or more efficient systems are equally valid for the purpose of reducing PM and UFP concentrations. During the activity, use personal protective equipment for handling the material itself (FFP3 mask, gloves). Avoid dust dispersion. Provide a good ventilation when handling. Avoid breathing dust. Keep away from sources of ignition. Graphite is an excellent electricity conductor; prevent dusts accumulation where such accumulations may cause short circuits
Other safety relevant observations:	Eye/face protection: Wear eye protection (complying with EN 166), if prevention measures are not sufficient to reduce the risk of eye contact. Hands and body protection: In case of prolonged or repeated contact with the skin, wear protective gloves of rubber or other material adequate to the specific tasks (complying with EN 374), and appropriate clothes. Respiratory protection: If the dusts dispersion cannot be adequately controlled by appropriate engineering controls, wear respiratory protective equipment, such as masks, half-masks or self-contained breathing apparatus (complying with EN 149, 140 or 136).

** According to the information provided by the sponsor, the test item is stable after two years*

Identification, concentration, composition and any other characteristic needed to define the test item were provided by the sponsor. The sponsor is responsible for the data provided or omitted concerning the characterisation of the test item.

Certificate of analysis is included in AnnexII.

9 EXPERIMENTAL DESIGN

The present study has been designed to evaluate the acute inhalation toxicity of the test item PURE G + in male and female Sprague Dawley rats by the traditional protocol described in the OECD Guideline Nº 403: Acute Inhalation Toxicity. This method provides lethality data of the test item and allows its classification according to the Globally Harmonized System (GHS) of Classification and Labelling of Chemicals.

The following table summarizes the different hazard categories according to GHS and their corresponding labels:

Category 1	Category 2	Category 3	Category 4	Category 5
Fatal if inhaled	Fatal if inhaled	Toxic if inhaled	Harmful if inhaled	May be harmful if inhaled

9.1 TREATMENT

The test was based on a stepwise procedure. The target starting dose was 2 mg/L air. According to the results from the technical trials, this concentration was found to be the highest technically achievable and respirable that could be maintained for at least 4 hours. The mean dose achieved during this study was 1.87 mg/L air.

A total of 3 male and 3 female rats (Group A, starting dose group) were exposed for 4 consecutive hours.

During an observation period of 14 days following the end of exposure, clinical observations and body weight were recorded in order to characterise the toxicological effects of the aerosol. At the end of this period, all surviving animals were subjected to a gross necropsy and descriptions of all macroscopic abnormalities were recorded.

As less than 50% lethality occurred at the maximum attainable concentration, no further testing was necessary.

9.2 EXPERIMENTAL GROUPS

Group	Dose (mg/L)	Exposure time (h)	Males ID	Females ID
A	1.87	4	1 - 3	4 - 6

9.3 EXPOSURE CONDITIONS

Inhalation exposure was performed using a flow-past, nose-only exposure system. The animals were confined separately in restraint tubes which were positioned radially around the exposure chamber. The exposure system ensured a uniform distribution and provided a constant flow of test material to each exposure tube. The mean flow of air at each tube was approximately 1.359 L/min, which was sufficient to minimize re-breathing of the test aerosol as it is more than twice the respiratory minute volume of rats.

Exposure chambers type EC-FPC-232 (anodised aluminium, volume inside compartment: approximately 3 L), equipped with glass exposure tubes were used. The rats were individually exposed in glass tubes matching their size. Before treatment start, the homogeneity for the different levels of the exposure chamber was confirmed. Cross and longitudinal sections of the chamber are shown in Figure 1.

The temperature and relative humidity of the test atmosphere in the exposure chamber were maintained as required by experimental conditions. Air flow was monitored regularly.

10 EXPERIMENTAL DATA

10.1 INHALATION TECHNICAL DATA

10.1.1 PREPARATION OF TEST ITEM

In order to facilitate the generation of a respirable aerosol, the test item was previously desiccated for about 45 hours.

10.1.2 GENERATION OF THE TEST ITEM ATMOSPHERE

A dust aerosol was generated from the desiccated test item using a Dust Generator SAG 410 (TOPAS GmbH, Germany). The dust was diluted with filtered air from a compressor and conveyed via glass tubing, from the generator to the exposure chamber (see Figure 2). The flow rate through the exposure chamber was adjusted as necessary.

10.1.3 TECHNICAL TRIALS

Technical trials were performed without animals and conducted before the in life phase of the study to establish the conditions for aerosol generation, which included:

- Determination of target concentration and/or technical limit. Several tests were performed to establish the highest stable aerosol concentration achievable that could be maintained at least for 4 hours. Initially, aerosol concentration was aimed to 5 mg/L air, but saturation and blockade of the system was observed shortly after starting. Finally, a stable aerosol of approximately 2 mg/L could be achieved with the TOPAS SAG 410 aerosol generator. Aerosol concentration was determined by gravimetric analysis.
- Determination of particle size distribution: Mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) were determined at the target concentration and calculated on the basis of the results from the cascade impactor, using Microsoft Excel® software (Microsoft Corporation, USA). The target ranges were 1 to 4 µm for the MMAD and 1.5 to 3 for the GSD. A respirable aerosol (MMAD in the range of 1-4 µm) could be achieved at 2 mg/L air. Therefore, starting dose was set at 2 mg/L although, as indicated above, the mean actual dose achieved during the present study was 1.87 mg/L.

10.1.4 PRE-CHARACTERISATION OF THE AEROSOL. SYSTEM SETUP

Previous to exposure onset, a complete setup of the system and characterisation of test atmosphere was performed according to the corresponding SOPs. The tasks were the following:

- Making an outline of each line including identification of the corresponding equipment.
- Leak test through the aerosol conveyor glass tubes.
- Leak test in dust generator.
- Identification of each group (name, dose, total number of animals, total flow of aerosol/air).
- Checking of air temperature and relative humidity.
- Theoretical calculations regarding dilution and Venturi tubes to be used.
- Vacuum system checking in sampling ports: capillary installed, vacuum pressure measurement, sampling flow.
- Exposure test: determination of pressure inside the inhalation chamber versus room pressure and difference of pressure between both chamber compartments. Determination of aerosol flow and aerosol concentration in different positions of the chamber randomly selected: six ports from the high level and six ports from the low level. Calculation of coefficient of variation (% CV) within each level and across the two levels. Within each level, the CV was not higher than 1.2 % (acceptance criteria 5 %), whereas the CV among the two levels did not exceed 5.5 % (acceptance criteria 10 %).

10.1.5 CHARACTERISATION OF THE TEST ITEM ATMOSPHERE DURING EXPOSURE

In order to characterise the test atmosphere and to check the reproducibility of aerosol generation and dilution, several analytical parameters were determined at defined intervals during exposure.

10.1.5.1 Determination of the nominal aerosol concentration

The test item usage was determined once per exposure by weighing the amount of the test item before and after exposure to determine the quantity used. The weight used was then divided by the total air-flow volume to give the nominal concentration. These data were used for the purpose of monitoring the performance of the generation system.

10.1.5.2 Gravimetric determination of the aerosol concentration

Gravimetric determination of the aerosol concentration was performed twice each hour of exposure. Test aerosol samples were collected onto a Whatman filter (grade F319.04) using a filter sampling device. The sampling flow was similar to the air flow rate per exposure port. The duration of sampling was 10 minutes. The filters were weighed before and immediately after sampling using a calibrated balance. The gravimetric aerosol concentration was calculated from the amount of test item present on the filter and the sample volume.

10.1.5.3 Determination of particle size distribution

The particle size distribution was determined gravimetrically twice during exposure. The cumulative particle size distribution of the test aerosol was determined using a PIXE cascade impactor. The particle size

distribution of the test item in the generated aerosol was measured by gravimetry analyzing the test item deposited on each stage of the cascade impactor.

The mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) were calculated on the basis of the results from the impactor, using Microsoft Excel® software (Microsoft Corporation, USA). The target ranges were 1 to 4 µm for the MMAD and 1.5 to 3 for the GSD.

10.1.5.4 Determination of the temperature

The temperature in the chamber was measured continuously during exposure using a thermohygrometer (TFA 30.5036.13). The target range was 19-25 ºC. The results were reported approximately hourly from the start of the inhalation exposure.

10.1.5.5 Determination of relative humidity

The relative humidity in the chamber was measured continuously during exposure using a thermohygrometer (TFA 30.5036.13). The ideal range recommended by the OECD guideline Nº 436 is 30-70 %, although it is also indicated that these values may be unattainable under certain conditions and this was the case of this study in which compressed air (dry air) was used to dilute the test item aerosol.

The results were reported approximately hourly from the start of the inhalation exposure.

10.1.5.6 Determination of oxygen and CO_2 concentration

The oxygen and CO_2 concentration of the test atmosphere were measured during the exposure period using a portable gas detector (PGAS-24-O2/CO2, PGAS-24). These concentrations were maintained above 19 % and below 1 % respectively. The results were reported approximately hourly from the start of the inhalation exposure.

10.1.5.7 Airflow rate

The exposure airflow rate was adjusted as appropriate before the start of the exposure using the pressure difference over a Venturi tube. The actual airflow rate was monitored at least hourly during exposure. The target range was 1.0 ± 0.5 L/min through each inhalation tube.

10.2 TEST SYSTEM DATA

Experimental data concerning animal group distribution, body weight, general clinical signs and gross necropsy findings were recorded through Provantis 8.5.2.1™ (Instem LSS Ltd., Staffordshire, United Kingdom) preclinical software.

10.2.1 IN LIFE OBSERVATIONS

10.2.1.1 Mortality and premature sacrifice

The animals were examined daily for mortality and morbidity. Any clinical signs, discomfort and mortality were recorded in accordance with the humane endpoints guidance document of the OECD.

10.2.1.2 Clinical observations

Clinical observations in response to treatment were performed on all animals hourly during exposure (only grossly abnormal signs), immediately and 1 h after exposure, and once daily thereafter until the end of the observation period. All animals were observed for a period of 14 days after administration. Any visible clinical signs and discomfort were recorded.

10.2.1.3 Body weight

All animals were weighed on the day of treatment just before starting exposure (study day 1), on study days 2, 4, 8 and immediately before sacrifice on study day 15.

10.2.2 TERMINAL INVESTIGATION

Animals were sacrificed at the end of the observation period by an intraperitoneal over dose of pentobarbital and subjected to a gross necropsy consisting in the examination of the abdominal and thoracic cavities and contents. Special attention was paid to any change in the respiratory tract.

11 STATISTICAL ANALYSIS

No statistical analysis was required.

12 STORAGE OF RECORDS AND ARCHIVE

Item	Storage conditions	Duration
Documents: General Study plan, Study-specific supplement and amendments Raw data Animal Facility records Test item storage room records Test item preparation records Final Report	Room Temperature	4 years

Final destination: after the storage period, items/documents will be made available to the sponsor, who will decide whether they will be sent back or further stored in the GLP archive (please note that a fee may be charged).

13 STUDY DEVIATIONS

The following study plan deviations were recorded:

Deviation 1

Mean temperature in the exposure chamber (25.9ºC) was slightly above the target range recommended by the OECD guideline Nº 403 (19 - 25 ºC). This incident is considered to have no impact on the quality / integrity of the study since it is highly unlikely that mortality, which is the main end point of the study, can be affected by this small and transient increase in temperature.

14 RESULTS

14.1 INHALATION TECHNICAL DATA

14.1.1 TEST ATMOSPHERE CONDITIONS

Mean temperature during exposure (25.9ºC) was slightly above the target range recommended by the OECD guideline Nº 403 (19 - 25 ºC).

Mean relative humidity of the aerosol during exposure was 24.2%, which was below of the range recommended by the guideline Nº 436 (30-70%) due to the fact that compressed air (dry air) was used to dilute the aerosol.

Data are presented in the following table:

Recording time (h:min from exposure start)	Temperature (ºC)	Relative humidity (%)
0:28	26.1	22.7
1:52	25.8	21.4
2:34	25.8	26.5
3:35	26.0	26.2
Mean	**25.9**	**24.2**
SD	**0.2**	**2.5**
N	**4**	**4**

Mean oxygen and carbon dioxide concentrations were 20.9 % and 0.08% respectively. These values are considered satisfactory for inhalation studies and within the target range (at least 19% and below 1% respectively).

Data are presented in the following table:

Recording time (h:min from exposure start)	Oxygen (%)	Carbon dioxide (%)
0:28	21.0	0.10
1:52	20.9	0.10
2:34	20.9	0.04
3:35	20.8	0.08
Mean	**20.9**	**0.08**
SD	**0.1**	**0.03**
N	**4**	**4**

Mean air flow rate through each inhalation tube was 1.359 L/min. This value is considered satisfactory for inhalation studies and within the target range (1.0 ± 0.5 L/min), since it is sufficient to clear the exhaled atmosphere away from the animal.

14.1.2 AEROSOL CONCENTRATIONS

14.1.2.1 Nominal aerosol concentration

The calculated nominal aerosol concentration was 2.04 mg/L air.

14.1.2.2 Gravimetric aerosol concentration

The mean of the gravimetric concentrations during exposure was 1.87 mg/L air (6.5 % deviation from target). Data on aerosol concentrations are presented in the following table:

Group A (Target: 2.0 mg/L air). Day 1 of study			
Sampling starting time (h:min from exposure start)	Sampling volume (L)	Amount of test item:PG (1:1) on the filter (mg)	Final Gravimetric aerosol concentration of test item (mg/L)
0:03	13.55	25.71	1.90
0:32	13.44	29.62	2.20
0:54	13.22	25.85	1.96
1:35	13.51	26.50	1.96
2:06	13.41	19.03	1.42
3:00	13.47	23.76	1.76
3:17	13.51	24.64	1.82
3:41	13.47	26.13	1.94
MEAN	**13.45**	**25.16**	**1.87**
SD	**0.10**	**3.01**	**0.22**
N	**8**	**8**	**8**

14.1.3 PARTICLE SIZE DISTRIBUTION (PSD)

Mean Mass Median Aerodynamic Diameter (MMAD) of particle size distribution during exposure was calculated from two gravimetric measurements PSD #1 and PSD #2. Mean MMAD during exposure was 1.010 µm. This value was within the target range (1-4 µm).

Geometric Standard Deviations (GSD) on PSD #1 and PSD #2 were 2.87 and 1.55, respectively, and therefore they were also within the target range (1.5 - 3.0).

In conclusion, the test item aerosol was considered to be respirable to rats and appropriate for acute inhalation toxicity testing.

Data on particle size distribution are presented in the following table:

		Particle size distribution test				
Stage #	Particle diameter range (µm)	PSD #1		PSD #2		Mean PSD
		% particles	Relative cumulative % particles	% particles	Relative cumulative % particles	Relative cumulative % particles
L1	0.06 - 0.12	0.00	0.00	0.00	0.00	0.00
L2	0.12 - 0.25	18.18	18.18	2.80	2.80	10.49
1	0.25 - 0.50	6.06	24.24	1.40	4.20	14.22
2	0.50 - 1.00	9.09	33.33	73.43	77.62	55.475
3	1.00 - 2.00	54.55	87.88	15.38	93.01	90.445
4	2.00 - 4.00	0.00	87.88	2.10	95.10	91.49
5	4.00 - 8.00	0.00	87.88	3.50	98.60	93.24
6	8.00 - 16.00	6.06	93.94	1.40	100.00	96.97
7	> 16.00	6.06	100.00	0.00	100.00	100.00

	Particle size distribution test		
	MMAD (µm)	GSD	% < 4 µm
PSD #1	1.250	2.87	87.88
PSD #2	0.770	1.55	95.10
Mean	**1.010**	-	**91.49**

14.2 TEST SYSTEM DATA

14.2.1 IN-LIFE OBSERVATIONS

14.2.1.1 Mortality

One male animal (ID 1) was found dead the day after exposure (study day 2). Upon gross necropsy, black spots were observed in the lungs and larynx, trachea, oesophagus and stomach were black colored. Furthermore, black contents in the stomach were also present.

These organs with findings were collected and preserved in neutral-buffered 4 % formaldehyde.

14.2.1.2 Clinical signs and observations

Individual data in Appendix 1.

Immediately after exposure, the male animal ID 1 presented dirty head fur (blackish), prostration, vocalization and breathing difficulty. This last sign was also observed during the four hours of exposure. One hour after finishing exposure these signs still remained and additionally piloerection and loud breathing were also observed. This animal was found dead the day after exposure (see Section 14.2.1.1).

The other two males also presented black fur on the head area which was visible until study day 8. Piloerection, prostration and breathing difficulty were observed in these animals 1 hour after finishing exposure but they were not present the day after. Cyanotic (bluish) hindlimbs were observed in animal ID 3 during exposure and immediately after, but this sign disappeared within the following hour. Respiratory crackles were recorded in this animal on study day 2 but it was no longer present during the following days.

In the three females, black fur on the head was also observed until study day 8. Additionally, transient piloerection was observed in one animal (ID 4) only 1 hour after finishing exposure.

All animals, regardless of sex, presented wet fur immediately after finishing the exposure period.

From study day 9 until the end of the 14 day-observation period, no clinical signs were observed in any of the five surviving animals and all of them exhibited a normal behavior.

14.2.1.3 Body weight

Individual data in Appendix 2 (body weight) and Appendix 3 (body weight gain).

A decrease in mean body weight of approximately 11% in males and 3% in females was observed between study day 1 (exposure) and study day 2.

From study day 2 to the end of the observation period (day 15), body weight increased gradually in all animals with the exception of the female ID 6 in which a small decrease (1 g, 0.5%) was observed between study days 4 and 8.

Total mean body weight gains of approximately 19% and 10% were recorded for males and females, respectively, during the 14 day observation period (from study day 1 to study day 15).

14.2.2 TERMINAL INVESTIGATION

14.2.2.1 Necropsy findings

Individual data in Appendix 4.

As it was mentioned in section 14.2.1.1 above, the male animal ID 1, found dead on study day 2, presented black spots in the lungs and black colored larynx, trachea, oesophagus and stomach. Furthermore, black contents in the stomach were also present.

In all the surviving animals (two males and three females) black spots were observed in the lungs.

The lungs were preserved in neutral-buffered 4 % formaldehyde.

15 DISCUSSION AND CONCLUSIONS

It can be concluded that, under the present experimental conditions:

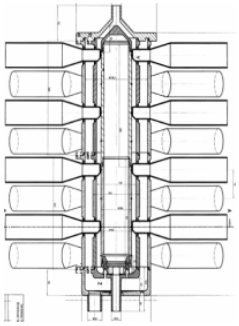
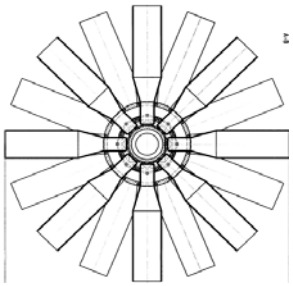
- The exposure of male and female Sprague Dawley rats to the test item PURE G + for 4 hours resulted in the premature death of one male animal.
- The respiratory effects of the test item were more evident in males than in females.
- The marginal body weight loss between study days 1 and 2 in all animals was considered to be mainly due to the stress related to the nose-only exposure procedure, although a contribution of the treatment with the test item cannot be excluded.
- The presence black spots in the lungs of all animals was considered to be related to test item exposure.
- The LC_{50} of PURE G + was greater than 1.87 mg/L air (gravimetric aerosol concentration).
- Based on the GHS classification criteria, PURE G + can be considered to be under human health hazard Category 4 (“harmful if inhaled”) or higher.

16 REFERENCES

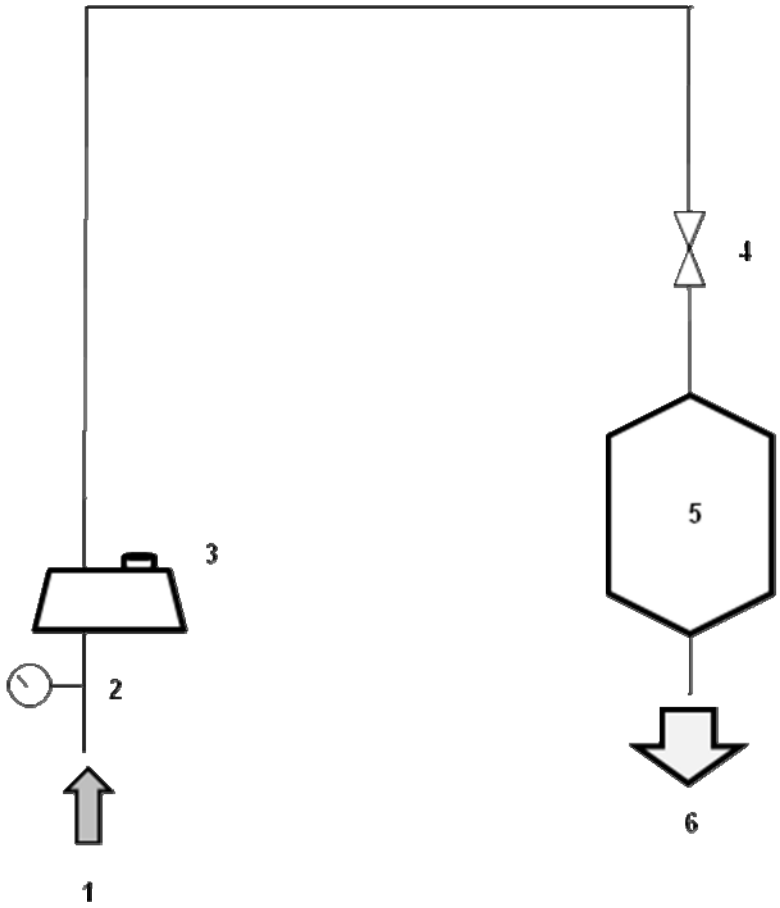
Code	Title
2004/10/EC	Good Laboratory Practices
RD 1369/2000	Principios de Buenas Prácticas de Laboratorio
OECD 403 (Adopted: 7 September 2009)	Acute Inhalation toxicity
OECD 39 (21 July 2009)	Guidance document on acute inhalation toxicity testing
Fifth revised edition, 2013	Globally Harmonized System (GHS) of Classification and Labelling of Chemicals

17 FIGURES

17.1 FIGURE 1. LONGITUDINAL AND CROSS SECTIONS OF THE EXPOSURE CHAMBER

Longitudinal section	Cross section
	

17.2 FIGURE 2. SCHEMATIC REPRESENTATION OF AEROSOL GENERATION AND INHALATION EXPOSURE SYSTEM



1. Supply of compressed air
2. Pressure controller
3. Aerosol generator (TOPAS SAG 410 Dust Generator)
4. Venturi tube to monitor air flow to the exposure chamber
5. Nose-only, flow-past exposure chamber as detailed in Figure 1
6. Exhaust of exposure chamber

18 APPENDICES

18.1 APPENDIX 1: INDIVIDUAL CLINICAL SIGNS

Appendix 1

Clinical observations throughout the study period. Individual data

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	1 1h exposure	1 2h exposure	1 3h exposure	1 Immediately postdose	1 1h post dose	2	3
A	m	1	Coat Soiled	Head	.	.	.	2	.	.	.
			Piloerection		.	.	.	.	I	.	.
			Vocalisation		.	.	.	2	2	.	.
			Found Dead		.	.	.	.	.	X	.
			dirty fur	Head	.	.	.	.	X	.	.
			Prostration		.	.	.	X	X	.	.
			loud breathing		.	.	.	.	X	.	.
			breathing difficulty		X	X	X	X	X	.	.
		2	No Abnormalities Detected		X	X	X	.	.	.	.
			Piloerection		.	.	.	.	I	.	.
			dirty fur	Head	.	.	.	X	X	X	X
			Prostration		.	.	.	.	X	.	.
			breathing difficulty		.	.	.	.	X	.	.
			Wet fur	Whole body	.	.	.	X	.	.	.
		3	No Abnormalities Detected		.	.	.	.	.	.	.
			Piloerection		.	.	.	.	I	.	.
			dirty fur	Head	.	.	.	X	X	X	X
			Prostration		.	.	.	.	X	.	.
			breathing difficulty		X	X	X	X	X	.	.
			Respiratory crackles		.	.	.	.	.	I	.
			Wet fur	Whole body	.	.	.	X	.	.	.
			Cyanotic	Left Hindlimb	X	X	X	X	.	.	.
			Cyanotic	Right Hindlimb	X	X	X	X	.	.	.

Severity Codes: X = Present; 2 = Moderate; I = Mild

Group A - A

Appendix 1

Clinical observations throughout the study period. Individual data

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	4	5	6	7	8	9	10
A	m	1	Coat Soiled	Head	.	.	.	.	.	.	.
			Piloerection		.	.	.	.	.	.	.
			Vocalisation		.	.	.	.	.	.	.
			Found Dead		.	.	.	.	.	.	.
			dirty fur	Head	.	.	.	.	.	.	.
			Prostration		.	.	.	.	.	.	.
			loud breathing		.	.	.	.	.	.	.
			breathing difficulty		.	.	.	.	.	.	.
		2	No Abnormalities Detected		.	.	.	.	.	X	X
			Piloerection		.	.	.	.	.	.	.
			dirty fur	Head	X	X	X	X	X	.	.
			Prostration		.	.	.	.	.	.	.
			breathing difficulty		.	.	.	.	.	.	.
			Wet fur	Whole body	.	.	.	.	.	.	.
		3	No Abnormalities Detected		.	.	.	.	.	X	X
			Piloerection		.	.	.	.	.	.	.
			dirty fur	Head	X	X	X	X	X	.	.
			Prostration		.	.	.	.	.	.	.
			breathing difficulty		.	.	.	.	.	.	.
			Respiratory crackles		.	.	.	.	.	.	.
			Wet fur	Whole body	.	.	.	.	.	.	.
			Cyanotic	Left Hindlimb	.	.	.	.	.	.	.
			Cyanotic	Right Hindlimb	.	.	.	.	.	.	.

Severity Codes: X = Present; 2 = Moderate; I = Mild

Group A - A

Appendix 1

Clinical observations throughout the study period. Individual data

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	11	12	13	14	15
A	m	1	Coat Soiled	Head	.	.	.	.	.
			Piloerection		.	.	.	.	.
			Vocalisation		.	.	.	.	.
			Found Dead		.	.	.	.	.
			dirty fur	Head	.	.	.	.	.
			Prostration		.	.	.	.	.
			loud breathing		.	.	.	.	.
			breathing difficulty		.	.	.	.	.
		2	No Abnormalities Detected		X	X	X	X	X
			Piloerection		.	.	.	.	.
			dirty fur	Head	.	.	.	.	.
			Prostration		.	.	.	.	.
			breathing difficulty		.	.	.	.	.
			Wet fur	Whole body	.	.	.	.	.
		3	No Abnormalities Detected		X	X	X	X	X
			Piloerection		.	.	.	.	.
			dirty fur	Head	.	.	.	.	.
			Prostration		.	.	.	.	.
			breathing difficulty		.	.	.	.	.
			Respiratory crackles		.	.	.	.	.
			Wet fur	Whole body	.	.	.	.	.
			Cyanotic	Left Hindlimb	.	.	.	.	.
			Cyanotic	Right Hindlimb	.	.	.	.	.

Severity Codes: X = Present; 2 = Moderate; I = Mild

Group A - A

Appendix 1

Clinical observations throughout the study period. Individual data

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	1 1h exposure	1 2h exposure	1 3h exposure	1 Immediately postdose	1 1h post dose	2	3
A	f	4	No Abnormalities Detected		X	X	X	.	.	.	.
			Piloerection		.	.	.	.	I	.	.
			dirty fur	Head	.	.	.	X	X	X	X
			Wet fur	Whole body	.	.	.	X	.	.	.
		5	No Abnormalities Detected		X	X	X	.	.	.	.
			dirty fur	Head	.	.	.	X	X	X	X
		6	No Abnormalities Detected		X	X	X	.	.	.	.
			dirty fur	Head	.	.	.	X	X	X	X
			Wet fur	Whole body	.	.	.	X	.	.	.

Severity Codes: X = Present; 2 = Moderate; I = Mild

Group A - A

Appendix 1

Clinical observations throughout the study period. Individual data

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	4	5	6	7	8	9	10
A	f	4	No Abnormalities Detected		.	.	.	.	.	X	X
			Piloerection		.	.	.	.	.	.	.
			dirty fur	Head	X	X	X	X	X	.	.
			Wet fur	Whole body	.	.	.	.	.	.	.
		5	No Abnormalities Detected		.	.	.	.	.	X	X
			dirty fur	Head	X	X	X	X	X	.	.
		6	No Abnormalities Detected		.	.	.	.	.	X	X
			dirty fur	Head	X	X	X	X	X	.	.
			Wet fur	Whole body	.	.	.	.	.	.	.

Severity Codes: X = Present; 2 = Moderate; I = Mild

Group A - A

Appendix 1

Clinical observations throughout the study period. Individual data

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	11	12	13	14	15
A	f	4	No Abnormalities Detected		X	X	X	X	X
			Piloerection		.	.	.	.	.
			dirty fur	Head	.	.	.	.	.
			Wet fur	Whole body	.	.	.	.	.
		5	No Abnormalities Detected		X	X	X	X	X
			dirty fur	Head	.	.	.	.	.
		6	No Abnormalities Detected		X	X	X	X	X
			dirty fur	Head	.	.	.	.	.
			Wet fur	Whole body	.	.	.	.	.

Severity Codes: X = Present; 2 = Moderate; I = Mild

Group A - A

18.2 APPENDIX 2: BODY WEIGHT. INDIVIDUAL VALUES

Appendix 2

Body weight throughout the study period. Individual values

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

Bodyweight (g)

			Day numbers relative to Start Date					
Group	Sex	Animal	-6	1	2	4	8	15
A	m	1	187.50	229.80	.	.	.	.
		2	188.00	227.30	204.40	215.50	231.90	274.50
		3	190.40	233.90	203.90	212.00	235.20	275.70
		Mean	188.633	230.333	204.150	213.750	233.550	275.100
		S.D.	1.550	3.332	0.354	2.475	2.333	0.849
		N	3	3	2	2	2	2

Appendix 2

Body weight throughout the study period. Individual values

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

Bodyweight (g)

Group	Sex	Animal	Day numbers relative to Start Date -6	1	2	4	8	15
A	f	4	165.70	193.70	184.50	192.10	196.90	216.40
		5	165.50	197.00	193.70	198.60	199.80	218.10
		6	164.80	191.20	184.30	188.50	187.50	207.40
		Mean	165.333	193.967	187.500	193.067	194.733	213.967
		S.D.	0.473	2.909	5.370	5.119	6.430	5.750
		N	3	3	3	3	3	3

18.3 APPENDIX 3: BODY WEIGHT GAIN. INDIVIDUAL VALUES

Appendix 3

Body weight gain throughout the study period. Individual values

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

				Bodyweight Gain (g)			
			From:	1	2	4	8
Group	Sex	Animal	To:	2	4	8	15
A	m	2		-22.90	11.10	16.40	42.60
		3		-30.00	8.10	23.20	40.50
			Mean	-26.450	9.600	19.800	41.550
			S.D.	5.020	2.121	4.808	1.485
			N	2	2	2	2

Appendix 3

Body weight gain throughout the study period. Individual values

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

Bodyweight Gain (g)

Group	Sex	Animal	From:	1	2	4	8
			To:	2	4	8	15
A	f	4		-9.20	7.60	4.80	19.50
		5		-3.30	4.90	1.20	18.30
		6		-6.90	4.20	-1.00	19.90
			Mean	-6.467	5.567	1.667	19.233
			S.D.	2.974	1.795	2.928	0.833
			N	3	3	3	3

18.4 APPENDIX 4: GROSS NECROPSY FINDINGS. INDIVIDUAL DATA

Appendix 4

Gross Necropsy Findings. Individual data

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

---

Animal Ref.: 1 Group: 1 Sex: Male Species: Rat Strain: Sprague Dawley

Test Material: See Protocol Dose: A Route: See Protocol Study Type: Inhalation
Date of Death : 04/26/18 Study Day No. (Week): 2 (1) Mode of Death: Found Dead
Date of Necropsy: 04/26/18 ** NECROPSY COMPLETE **

Last Clinical Observations:

Dirty fur; Head
Prostration
Loud breathing
Breathing difficulty

Palpable Mass Details:

None

Terminal Body Weight: None

Gross Pathology Observations:

-Lungs: Black spots. -Trachea, Larynx, oesophagus, stomach: Black coloured and black contents.

Any remaining protocol required tissues, which have been examined, have no visible lesions

Probable cause of death: None

Appendix 4

Gross Necropsy Findings. Individual data

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

---

Animal Ref.: 2 Group: 1 Sex: Male Species: Rat Strain: Sprague Dawley

Test Material: See Protocol Dose: A Route: See Protocol Study Type: Inhalation
Date of Death : 05/09/18 Study Day No. (Week): 15 (3) Mode of Death: Killed Terminal
Date of Necropsy: 05/09/18 ** NECROPSY COMPLETE **

Last Clinical Observations:

None

Palpable Mass Details:

None

Terminal Body Weight: 274.5g

Gross Pathology Observations:

LUNGS;
Black Spots (Left)
Black Spots (Right)

Any remaining protocol required tissues, which have been examined, have no visible lesions

Probable cause of death: None

Appendix 4

Gross Necropsy Findings. Individual data

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

---

Animal Ref.: 3 Group: 1 Sex: Male Species: Rat Strain: Sprague Dawley

Test Material: See Protocol Dose: A Route: See Protocol Study Type: Inhalation
Date of Death : 05/09/18 Study Day No. (Week): 15 (3) Mode of Death: Killed Terminal
Date of Necropsy: 05/09/18 ** NECROPSY COMPLETE **

Last Clinical Observations:

None

Palpable Mass Details:

None

Terminal Body Weight: 275.7g

Gross Pathology Observations:

LUNGS;
Black Spots (Left)
Black Spots (Right)

Any remaining protocol required tissues, which have been examined, have no visible lesions

Probable cause of death: None

Appendix 4

Gross Necropsy Findings. Individual data

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

---

Animal Ref.: 4 Group: 1 Sex: Female Species: Rat Strain: Sprague Dawley

Test Material: See Protocol Dose: A Route: See Protocol Study Type: Inhalation
Date of Death : 05/09/18 Study Day No. (Week): 15 (3) Mode of Death: Killed Terminal
Date of Necropsy: 05/09/18 ** NECROPSY COMPLETE **

Last Clinical Observations:

None

Palpable Mass Details:

None

Terminal Body Weight: 216.4g

Gross Pathology Observations:

LUNGS;
Black Spots (Left)
Black Spots (Right)

Any remaining protocol required tissues, which have been examined, have no visible lesions

Probable cause of death: None

Appendix 4

Gross Necropsy Findings. Individual data

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

Animal Ref.: 5 Group: 1 Sex: Female Species: Rat Strain: Sprague Dawley

Test Material: See Protocol Dose: A Route: See Protocol Study Type: Inhalation
Date of Death : 05/09/18 Study Day No. (Week): 15 (3) Mode of Death: Killed Terminal
Date of Necropsy: 05/09/18 ** NECROPSY COMPLETE **

Last Clinical Observations:

None

Palpable Mass Details:

None

Terminal Body Weight: 218.1g

Gross Pathology Observations:

LUNGS;
Black Spots (Left)
Black Spots (Right)

Any remaining protocol required tissues, which have been examined, have no visible lesions

Probable cause of death: None

Appendix 4

Gross Necropsy Findings. Individual data

B-02523 - Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

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Animal Ref.: 6 Group: 1 Sex: Female Species: Rat Strain: Sprague Dawley

Test Material: See Protocol Dose: A Route: See Protocol Study Type: Inhalation
Date of Death : 05/09/18 Study Day No. (Week): 15 (3) Mode of Death: Killed Terminal
Date of Necropsy: 05/09/18 ** NECROPSY COMPLETE **

Last Clinical Observations:

None

Palpable Mass Details:

None

Terminal Body Weight: 207.4g

Gross Pathology Observations:

LUNGS;
Black Spots (Left)
Black Spots (Right)

Any remaining protocol required tissues, which have been examined, have no visible lesions

Probable cause of death: None
---

19 ANNEXES

19.1 ANNEX I: VIVOTECNIA GLP CERTIFICATE

ENAC
Entidad Nacional de Acreditación

Evaluada la conformidad de BPL de acuerdo con la Directiva 2004/9/EC y el Real Decreto 2043/1994
Verified the compliance of GLP according to the Directive 2004/9/EC and Royal Decree 2043/1994

Otorga el presente
Grants this Certificate of Compliance with Good Laboratory Practice

CERTIFICADO DE CUMPLIMIENTO DE BUENAS PRÁCTICAS DE LABORATORIO

17/41/BPL57

a la entidad de ensayo / to the test facility

VIVOTECNIA RESEARCH, S.L.
C/ Santiago Grisolia, 2 28760 Tres Cantos (MADRID)

La entidad lleva a cabo estudios de **sustancias químicas** en laboratorio, en las áreas de ensayo de: **toxicidad(2*)** y **mutagenicidad(3*)** en cumplimiento con los principios de BPL, tal como ha establecido la Unión Europea y la OCDE.
The test facility performs studies with **chemical substances** in laboratory, in the areas of expertise: **toxicity(2*)** and **mutagenicity(3*)** in compliance with GLP principles established by EU and OECD

Fecha de entrada en vigor / Coming into effect: 06/10/2017
Validez hasta / Valid until: 06/10/2019
Fecha de inspección / Date of inspection: 14, 15 y 16/06/2017
Certificada por primera vez el / Certified since: 25/09/2015

D. José Manuel Prieto Barrio
Presidente
6 de octubre de 2017

* áreas de ensayo según Decisión OCDE C(89)87
* areas of expertise according to the Decision OCDE C(89)87

19.2 ANNEX II: CERTIFICATE OF ANALYSIS FOR TEST ITEM

DIRECTAPLUS
PARTNERS IN NANOTECHNOLOGY

Certificate of Analysis

PRODUCT INFORMATION:

Product: Pure G+

Lot number: P219/2016

Appearance: Black powder

Date of the analysis: 18/10/2016

Order number: -

QUALITY INFORMATION:

Parameter	*Value**	*Unit*	*Directa Plus Test Method*	*Acceptance criteria*
Apparent density	*40.1*	*g/L*	*A001*	*40 ± 10*
pH	*6.63*	*-*	*A005*	*6 ± 1.5*
Ash	*0.16*	*%*	*A040*	*<2*
Moisture	*0.13*	*%*	*A040*	*<1*
Particle size distribution (Dx50)	*4.39*	*µm*	*A050*	*4 ± 2*

** Measured values*

Date

19/02/2018

Reviewed by

Firmato da:
VOLONTE' CAROLINA
Motivo:
Responsible of Quality Cont rol

Data: 19/02/2018 16:19:28

The results contained in this Certificate of Analysis refer only to the sample tested.

This Certificate of Analysis may not be reproduced in partial form without the written permission of Directa Plus SpA. The laboratory applies quality management practices in accordance with the provisions of the standard UNI CEI EN ISO 9001:2015 e 14001:2015.

Document with digital signature in accordance with current legislation.
DP-PPROD-15 Rev. 0.0

19.3 ANNEX III: CERTIFICATE OF ANALYSIS FOR DIET AND DRINKING WATER

2914C

Teklad Certified Irradiated Global 14% Protein Rodent Maintenance Diet

Batch Number	**2914C-110617MA**
Certificate Number	17-N12905
Date of Manufacture	November 6, 2017
Expiry Date	August 3, 2018

Certificate of Analysis

			Action Limits				
Nutrients	**Result**		**Min**	**Max**	**Status**	**LOD**	**Lab SOP**
Moisture	**12.27**	%		13.0		0.1%	AACC 44-19
Protein (N x 6.25)	**14.0**	%	12.5	15.0		0.1%	AOAC 990.03
Oil (Acid Hydrolysis)	**4.47**	%	2.0	6.0		0.01%	AOAC 954.02
Crude Fibre	**3.86**	%	1.0	4.5		0.5%	AOAC 962.09
Ash	**4.42**	%	2.5	5.0		0.1%	AOAC 942.05
Calcium	**0.7**	%	0.5	1.0		100 mg/kg	AOAC 968.08
Phosphorus	**0.63**	%	0.4	0.8		625 mg/kg	AOAC 965.17
Magnesium	**0.26**	%	0.15	0.35		100 mg/kg	AOAC 968.08
Sodium	**0.1**	%	0.05	0.2		0.06 %	AOAC 968.08
Potassium	**0.57**	%	0.3	0.9		600 mg/kg	AOAC 968.08
Chloride	**0.25**	%	0.1	0.5		0.02 %	AOAC 969.10
Zinc	**70.38**	mg/kg	55.0	95.0		0.5 mg/kg	AOAC 968.08
Manganese	**90.56**	mg/kg	60.0	150.0		0.25 mg/kg	AOAC 968.08
Copper	**12.3**	mg/kg	8.0	20.0		0.25 mg/kg	AOAC 968.08
Iron	**152.8**	mg/kg	100.0	250.0		0.75 mg/kg	AOAC 968.08
Vitamin A	**5.68**	iu/g	4.0	12.0		0.6 iu/g	AOAC 974.29
Vitamin E	**97.5**	iu/kg	50.0	120.0		0.2 iu/g	AOAC 971.30
Contaminants						**LOD (mg/kg)**	
Nitrate	**nd**	mg/kg		100.0		4.0	AOAC 968.07
Nitrite	**0.36**	mg/kg		5.0		0.4	AOAC 968.07
Selenium	**0.22**	mg/kg		0.5		0.02	SW846 7741
Arsenic	**0.07**	mg/kg		1.0		0.025	SW846 6020
Cadmium	**0.07**	mg/kg		0.4		0.005	SW846 6020
Lead	**0.16**	mg/kg		3.0		0.025	SW846 6020
Mercury	**nd**	mg/kg		0.1		0.03	SW846 7473
Fluoride	**6.9**	mg/kg		40.0		15-30	AOAC 975.08
Total DDT	**nd**	mg/kg		0.1		0.03	CDFA MSP
Dieldrin	**nd**	mg/kg		0.02		0.02	CDFA MSP
Lindane	**nd**	mg/kg		0.1		0.03	CDFA MSP
Heptachlor	**nd**	mg/kg		0.05		0.02	CDFA MSP
Malathion	**nd**	mg/kg		0.5		0.05	CDFA MSP
Pirimiphos methyl	**nd**	mg/kg		5.0		0.05	CDFA MSP
Other residues*	**nd**	mg/kg		ns		*	CDFA MSP
Total PCBs	**nd**	mg/kg		0.25		0.01	CDFA MSP
Total Aflatoxins	**nd**	µg/kg		5.0		0.0005	Veratox HS
Microbiology							
Total Viable Count	**nd**	cfu/g		0.0		<10 cfu/g	BAM 8th Ed
Mesophilic Spores	**nd**	cfu/g		0.0		<10 cfu/g	AMM 329
Salmonella	**nd**			0.0		nf in 25g	AOAC BAM 2004.03
Coliforms	**nd**	cfu/g		0.0		10 cfu/g	BAM 8th Ed
E.coli	**nd**	cfu/g		0.0		ng in 1/g	BAM 8th Ed
Fungal Spores	**nd**	cfu/g		0.0		nf in 10g	BAM 8th Ed
Antibiotic Activity	**nd**			0.0		na	AMM/358

Data entered by: Rebecca Porter 2017.11.30 13:42:17 -06'00'

Data checked by: 2017.12.07 17:40:28 -06'00'

nd - None detected (see the level of detection limit, LOD)
Unless otherwise stated results on this certificate are expressed on a 'as received' moisture basis
* See attached list for the other residues tested
Recoveries for PCBs and Residues are 70 - 120%

Page 1 of 2

Envigo Teklad Diets + Madison WI + envigo.com + tekladinfo@envigo.com + (800) 483-5523

2914C

ENVIGO

Teklad Certified Irradiated Global 14% Protein Rodent Maintenance Diet

Batch Number	**2914C-110617MA**
Certificate Number	17-N12905
Date of Manufacture	November 6, 2017
Expiry Date	August 3, 2018

Multi-Residue Screen

Organophosphorus	Level (mg/kg)	LOD
Acephate	nd	0.1
Azinphos Methyl	nd	0.2
Bromophos Ethyl	nd	0.08
Bromophos	nd	0.05
Carbophenothion	nd	0.07
Chlorfenvinphos	nd	0.1
Chlorpyrifos	nd	0.04
Chlorpyrifos Methyl	nd	0.05
Demeton-S-Methyl	nd	0.1
Diazinon	nd	0.05
Dichlorvos	nd	0.06
Dimethoate	nd	0.1
Ethion	nd	0.07
Ethopyrophos	nd	0.05
Etrimfos	nd	0.05
Fenchlorphos	nd	0.1
Fenitrothion	nd	0.06
Fenthion	nd	0.06
Fonophos	nd	0.03
Heptenophos	nd	0.1
Malathion	nd	0.05
Methamidophos	nd	0.2
Methidathion	nd	0.1
Mevinphos	nd	0.05
Omethoate	nd	0.1
Parathion	nd	0.03
Parathion Methyl	nd	0.03
Phosalone	nd	0.1
Phosmet	nd	0.1
Phosphamidon	nd	0.06
Pirimiphos Methyl	nd	0.05
Quinalphos	nd	0.05
Tolclofos Methyl	nd	0.05
Triazophos	nd	0.05

Organochlorine	Level (mg/kg)	LOD
Aldrin	nd	0.02
Chlordane	nd	0.07
DDT	nd	0.03
Dieldrin	nd	0.02
Endosulphan	nd	0.03
Endrin	nd	0.05
Heptachlor	nd	0.02
Hexachlorobenzene (HCB)	nd	0.02
Hexachlorocyclohexane - a	nd	0.03
Hexachlorocyclohexane - b	nd	0.08
Hexachlorocyclohexane - g	nd	0.02
PCA	nd	0.05
Quintozene	nd	0.05
Tecnazene	nd	0.05

Pyrethroids		
Bifenthrin	nd	0.1
Cyfluthrin	nd	0.1
lamda Cyhalothrin	nd	0.1
Cypermethrin	nd	0.1
Deltamethrin	nd	0.1
Fenvalerate	nd	0.1
Flucythrinate	nd	0.1
Permethrin	nd	0.5
Tefluthrin	nd	0.05

Triazines		
Atrazine	nd	0.1
Cyanazine	nd	0.1
Prometryn	nd	0.1
Simazine	nd	0.1
Terbuthylazine	nd	0.1
Terbutryn	nd	0.1

Dicarboximides	Level (mg/kg)	LOD
Iprodione	nd	0.2
Procymidone	nd	0.1
Vinclozolin	nd	0.05

Organonitrogen		
Chlorpropham	nd	0.5
Etridiazole	nd	0.1
Fenarimol	nd	0.08
Metalaxyl	nd	0.2
Metribuzin	nd	0.2
Pirimicarb	nd	0.2
Propachlor	nd	0.3
Propham	nd	0.2
Propiconazole	nd	0.3
Propyzamide	nd	0.1
Thiabendazole	nd	1.0
Tolyfluanid	nd	0.07
Triadimefon	nd	0.2
Triadimenol	nd	0.1
Trifluralin	nd	0.03

Others		
Cadusafos	nd	0.05
Chlorothalonil	nd	0.2
Chlorthal-dimethyl	nd	0.05
Chlorthion	nd	0.05
Dichlofluanid	nd	0.05
Dicofol	nd	0.1
Diphenylamine	nd	0.1
Methoxychlor	nd	0.08
Pendimethalin	nd	0.05
Pentachlorobenzene (PCB)	nd	0.5
Tetradifon	nd	0.05
Tebuconazole	nd	0.3

Envigo Teklad Diets + Madison WI + envigo.com + tekladinfo@envigo.com + (800) 483-5523

eurofins | Eurofins Anàlisis Alimentario SL

Analytical report

Sample code Nr.	**386-2017-00063703**	**Report Date**	**22/09/2017**	**Page 1/1**
Analytical Report Nr.	**AR-17-AQ-060874-01 / 386-2017-00063703**			

VIVOTECNIA RESEARCH SL

For the attention of **Raúl Lloret Banderas**
C/ SANTIAGO GRISOLIA, 2
28760 TRES CANTOS
ESPAÑA
Fax 917280712
Email lloret@vivotecnia-ms.com

Copy to : Lloret Banderas (lloret@vivotecnia-ms.com)

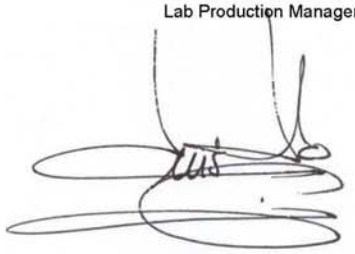
Your contact for Customer Service :

Our reference :	386-2017-00063703/ AR-17-AQ-060874-01	**Type :**	EX
Sample described as :	AGUA GRIFO AA9		
Sample reception date :	19/09/2017	**Analysis starting date :**	19/09/2017
Product temperature (°C)	22.6		

Microbiology	Results
UMPHK AQ Culturable Microorganisms 22°C E (Water) <100 >300000 /ml (2-3) PCA Agar-P ISO 6222-M Method : C5126245 (Recuento en placa:PCA)	
Culturable Microorganisms 22°C	< 100 cfu/ml
UMREG AQ Coliforms-Escherichia coli E (Water) <1 >100 /100 ml (0) CCA Agar-F ISO 9308-1-M Method : C5126246/C5126251 (Recuento por filtración en membrana:CCA)	
Coliforms	< 1 cfu/100 ml
Escherichia coli	< 1 cfu/100 ml

SIGNATURE

Luis Portillo
Lab Production Manager



Microbiology validated by Luis Portillo

Report electronically validated by Luis Portillo

EXPLANATORY NOTE

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Results have been obtained and reported in accordance with our general sales conditions available on request.
When declaring compliance or non-compliance, the uncertainty associated with the result has been added or subtracted in order to obtain a result that can be compared to regulatory limits or specifications.The uncertainty has not been taken into account for standards that already include measurement uncertainty or on explicit request of client.
The tests are identified by a five-digit code, their description is available on request.
NE: The term "estimated number" means a less accurate estimate of the true value when plate counts are less than 10 colonies.

The tests identified by the two letters code AQ are performed in laboratory Eurofins Anàlisis Alimentario SL.
- ACCREDITATION in compliance with UNE EN ISO/IEC 17025:2005 ENAC nº 1094/LE2182.

Eurofins Anàlisis Alimentario SL
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foodtestingmadrid@eurofins.com
www.eurofins.es

Marked activities with (*) are not covered by the accreditation of ENAC.

ENAC ENSAYOS nº 1094/LE2182

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Analytical report

Sample code Nr.	**386-2017-00063704**	**Report Date**	**20/09/2017**	**Page 1/2**
Analytical Report Nr.	**AR-17-AQ-060441-01 / 386-2017-00063704**			

VIVOTECNIA RESEARCH SL

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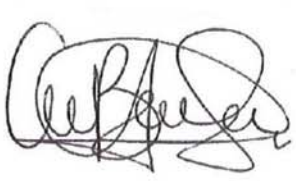
Your contact for Customer Service :

Our reference :	386-2017-00063704/ AR-17-AQ-060441-01	**Type :**	EX
Sample described as :	AGUA GRIFO AA9		
Sample reception date :	19/09/2017	**Analysis starting date :**	19/09/2017
Product temperature (°C)	22.6		

Chemistry		Results (uncertainty)	Guidelines
AQ022	**AQ Ammonium Method : Internal Method, Spectrophotometry (UV/VIS)**		
(*)	Ammonium	ND<0.05 mg/l	<=0.50(Guia) (RD140/2003)
AQ049	**AQ Colour Method : Internal Method, Spectrophotometry (UV/VIS)**		
(*)	colour	5 mg Pt/l	<=15(Guia) (RD140/2003)
AQ052	**AQ Conductivity Method : Internal Method C5127239 Conductimetry, Conductivity (membrane)**		
	Conductivity	121 (± 11) µS/cm	<=2500(Guia) (RD140/2003)
AQ110	**AQ Chlorine determination Method : Internal Method, Spectrophotometry (UV/VIS)**		
(*)	Combined chlorine	0.3 mg/l	<=2 (RD140/2003)
(*)	Free chlorine	ND<0.1 mg/l	<=1 (RD140/2003)
(*)	Total chlorine	0.3 mg/l	
AQ121	**AQ Nitrites (water) Method : Internal Method C5127241 Spectrophotometry, Spectrophotometry (UV/VIS)**		
	Nitrite (as NO2)	0.45 (± 0.06) mg NO2/l	<= 0.5 (RD140/2003)
AQ129	**AQ pH (20ºC) Method : Internal Method C5127238 Potentiometry, Potentiometry**		
	pH	7.4 (± 0.2)	6.5-9.5(Guia) (RD140/2003)
AQ146	**AQ Flavour Method : Internal Method, Organoleptic**		
(*)	Flavour	0	<=3(Guia) (RD140/2003)
AQ160	**AQ Turbidity Method : Internal Method C5127237 Nephelometry, Refractometry**		
	Turbidity	0.14 (± 0.01) NFU	<=5(Guia) (RD140/2003)
AQ227	**AQ Smell Method : Internal Method Sensory Analysis, Organoleptic**		
(*)	Smell	0	<=3(Guia) (RD140/2003)

SIGNATURE

Cristina Bernal
ASM Laboratory



Chemistry validated by Cristina Bernal

Report electronically validated by Cristina Bernal

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Analytical report

Sample code Nr.	**386-2017-00063704**	**Report Date**	**20/09/2017**	**Page 2/2**
Analytical Report Nr.	**AR-17-AQ-060441-01 / 386-2017-00063704**			

EXPLANATORY NOTE
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When declaring compliance or non-compliance, the uncertainty associated with the result has been added or subtracted in order to obtain a result that can be compared to regulatory limits or specifications.The uncertainty has not been taken into account for standards that already include measurement uncertainty or on explicit request of client.
The tests are identified by a five-digit code, their description is available on request.

The tests identified by the two letters code AQ are performed in laboratory Eurofins Anàlisis Alimentario SL.
- ACCREDITATION in compliance with UNE EN ISO/IEC 17025:2005 ENAC nº 1094/LE2182.

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ENAC ENSAYOS nº 1094/LE2182

19.4 ANNEX IV: GENERAL STUDY PLAN AND STUDY SPECIFIC SUPPLEMENT

vivotecnia
researching for you

GENERAL STUDY PLAN (GSP)

Acute inhalation toxicity in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

Test facility
VIVOTECNIA Research S.L.
Science to Business
C/Santiago Grisolía, 2 (PTM)
28760 Tres Cantos, Madrid
Spain

CONFIDENTIAL

CONTENTS

1 ORGANISATION AND RESPONSIBLITIES

Test facility

Vivotecnia Research S.L.
C/Santiago Grisolía, 2
28760 Tres Cantos
Madrid, Spain

Study Director

Dr. Pilar Prieto Arroyo
Tel: +34 91 728 07 10
e-mail: prieto@vivotecnia.com

Quality Assurance Unit Director

María Herrero Conde
Tel: +34 91 728 07 14
e-mail: herrero@vivotecnia.com

2 DECLARATIONS AND SIGNATURES

Study director

12.03.2018
Dr. Pilar Prieto
Date and signature

I hereby declare that the present study will be conducted according to the Good Laboratory Practice (GLP) requirements of the European Directive 2004/10/EC and the Spanish law RD 1369/2000, with the exception of technical trials to establish the conditions for aerosol generation which will be performed before the study initiation date and are therefore, excluded from GLP compliance.

Laboratory director

12.03.18
Dr. Andrés Konig
Date and signature

I hereby declare that I have read and approved the present study plan. A sufficient number of qualified personnel, appropriate facilities, equipment, and materials will be available to ensure that the study is conducted in an appropriate and timely manner.

Quality assurance

12.03.18
María Herrero
Date and signature

The present study will be conducted according to the Good Laboratory Practice (GLP) requirements of the European Directive 2004/10/EC and the Spanish law RD 1369/2000.

- The present study plan has been audited by Vivotecnia's quality assurance unit (QAU).
- QAU will survey on site critical phases of the study according to pre-determined schedules. Raw data generated throughout the study will be audited by QAU.
- The final report will be audited by QAU.

2.1 Adherence to protocol

2.1.1 Amendments to the study plan

- Amendments to the study plan will be approved by the study director and study monitor before implementation.
- Deviations to the study plan will be documented at the test facility and will be acknowledged by the study director. Any deviation will be reported to the study monitor as soon as possible and will be documented in the final report.
- The study director will assess the impact of the deviations on the quality and / or integrity of the study.

2.1.2 Means of communication

- Communication between the study director and the study monitor will be in written form (e-mail).

2.2 Regulatory compliance

- The present study will be conducted according to the Good Laboratory Practice (GLP) requirements of the European Directive 2004/10/EC and the Spanish law RD 1369/2000.
- Technical trials to establish the conditions for aerosol generation will be conducted using established procedures based on GLP, but carried out before the study initiation date. Therefore this part will be excluded from the statement of compliance.

3 STUDY SCHEDULE

The study schedule will be detailed in the study-specific supplement. A draft final report will be issued within 6 weeks after last necropsy or within 8 weeks if analytical chemistry needs to be performed.

4 OBJECTIVE

The objective of this study is to evaluate the acute inhalation toxicity of a test item in male and female Sprague Dawley rats by the Traditional Protocol described in the OECD Guideline Nº 403: Acute Inhalation Toxicity. This method has been designed to obtain sufficient information on the acute toxicity of the test item to enable its classification according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and to provide lethality data.

5 TEST SYSTEM AND CONDITIONS

5.1 Test system characterisation

Species:	Rat
Strain:	Hsd: Sprague Dawley® SD®
Sex:	Male
Colour:	*Albino*
Supplier:	Envigo RMS Spain S.L. Ctra. St. Miquel del Fai, Km. 3.5 08182 - Sant Feliú de Codines Barcelona – Spain
Health status*:	Specific Pathogen Free (SPF). Females will be nulliparous and non-pregnant
Rationale for selection of species / strain:	The Sprague Dawley rat is a suitable rodent strain, acceptable to regulatory authorities as a recommended rodent test system, and for which extensive background data are available
Total number of animals:	Limit test: 6 animals in a single group (3 males and 3 females). or Main study: 10 animals (5 males and 5 females or 5 animals of the susceptible sex, if known) per concentration level, with at least three concentration levels
Age of the animals at exposure:	8 - 12 weeks
Animals per cage:	3 or 5 of the same sex depending of the study (Limit test or Main study)
Bedding material:	Sodispan (SR-CHOPO-T) (autoclavable)
Change of cages:	At least once a week and when deemed necessary throughout the study period
Inclusion criteria on arrival:	Veterinary inspection
Acclimatisation period:	Animals will be acclimatised to Vivotecnia housing facilities at least 5 days prior exposure. Acclimatisation to the nose-only restraining tubes will be performed for at least 30 minutes the day of exposure.
Animal identification:	Digit ink marks

**The health monitoring report provided by the animal supplier will be stored at Vivotecnia.*

5.2 Animal facilities

Location:	Vivotecnia Research animal facilities C/Santiago Grisolía, 2 28760 Tres Cantos (Madrid) Spain
Accreditation authority:	Dirección General de Agricultura, Comunidad de Madrid (ES 289030000025)
Applicable law:	European Directives 2010/63/UE and Spanish Law RD 53/2013
Target parameters*	
Light cycle:	12h light : 12h dark
Pressure gradient:	Animal husbandry room positive to corridors
Temperature:	22 ± 3ºC
Relative humidity:	30 – 70%

**Environmental parameters will be classified, evaluated, recorded and reported on a daily basis according to the internal SOPs.*

5.3 Animal welfare

Animals showing continuous signs of severe distress and/or pain will be humanely sacrificed (in accordance with Vivotecnia's SOP following Institute for Laboratory Animal Research (ILAR) guideline: Humane endpoints for animals used in biomedical research and testing), and will be considered in the interpretation of the study results in the same way as animals that died on test.

Whenever possible animals will be kept in groups. Enrichment devices (nesting material, tubes, and chew blocks) will be provided as a default husbandry practice.

5.4 Animal diet

Food:	Global diet
Reference:	2914C
Supplier:	Harlan Teklad Station Road Blackthorn, Bicester Oxon, OX25 1TP United Kingdom
Nutritional / contaminant contents:	Certificate of analysis for the batch used in the study will be included in the final report
Food availability:	*Ad libitum*
Drinking water:	Tap water
Watering:	Bottles
Quality control*:	Certificate of analysis will be included in the final report
Water availability:	*Ad libitum*

**Contaminants in the diet / drinking water are considered not to be present at levels at which they might interfere with the quality and objectives of the study.*

6 EXPERIMENTAL DESIGN

The present study has been designed to evaluate the acute inhalation toxicity of a test item in male and female Sprague Dawley rats by the traditional protocol described in the OECD Guideline Nº 403: Acute Inhalation Toxicity. This method will provide lethality data of the test item and will allow its classification according to the Globally Harmonized System (GHS) of Classification and Labelling of Chemicals.

In the traditional protocol, groups of animals will be exposed to a test item for a fixed period of time (generally 4 hours) in a nose-only exposure chamber. Animals will be exposed to either a limit concentration (limit test) or to at least three concentrations in a stepwise procedure (main study). A sighting study may precede a main study unless some information about the test item already exists, such as a previously performed OECD 436 study.

In all cases, mortality, clinical observations and body weight will be recorded during an observation period of at least 14 days in order to characterise the toxicological effects of the test atmosphere. At the end of the observation period, all animals will be subjected to a gross necropsy and all gross pathological changes will be recorded.

6.1 Inclusion criteria

Only animals that meet the inclusion criteria will be recruited for the study and distributed into experimental groups.

Inclusion criteria:	20% of the mean body weight and in good health status.
Stratification method:	During the acclimatisation period, animals will be randomly distributed by means of the body weight stratification method.

6.2 Treatment

The test is based on a design in which groups of animals are exposed to either a limit concentration (limit test) or a series of concentrations in a stepwise procedure (main test) for a predetermined duration of usually 4 hours.

Limit test: A limit test is used when the test article is known or expected to be virtually non-toxic, i.e. eliciting a toxic response only above the regulatory limit concentration. In a limit test, a single group of three males and three females is exposed to the test item at a limit concentration. The starting dose will be selected based on regulatory requirements (in GHS Classification System, the limit concentration for aerosols is 5 mg/L) and on the results from technical trials. Decision on exposing further animals to additional doses according to the OECD test guideline Nº403 will be based on the mortality or morbidity observed at the limit test. If less than 50% lethality occurs at the maximum attainable concentration, no further testing is necessary.

Main study: A main study is typically performed using five males and five females (or 5 animals of the susceptible sex, if known) per concentration level, with at least three concentration levels. The time interval between exposure groups is determined by the onset, duration, and severity of toxic signs. Exposure of animals at the next concentration level should be delayed until there is reasonable confidence of survival for previously tested animals.

When testing aerosols, the primary goal should be to achieve a respirable particle size.

Lost exposure time (downtime), due for instance to technical reasons, will be compensated for at the end of exposure, as appropriate.

6.3 Exposure conditions

Inhalation exposure will be performed using a flow-past, nose-only exposure system. The animals will be confined separately in restraint tubes which are positioned radially around the exposure chamber. The exposure system ensures a uniform distribution and provides a constant flow of test material to each exposure tube. The flow of air at each tube will be 1 ± 0.5 L/min, which is sufficient to minimize re-breathing of the test aerosol as it is more than twice the respiratory minute volume of rats.

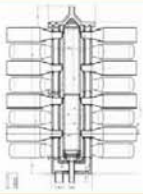
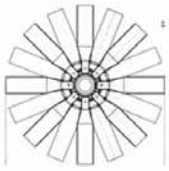
Exposure chambers type EC-FPC-232 (anodized aluminium, volume inside compartment: approximately 3 L), equipped with glass exposure tubes will be used. The rats will be individually exposed in glass tubes

matching their size. Before treatment start the homogeneity for the different levels of the exposure chamber will be confirmed.

The temperature and relative humidity of the test atmosphere in the exposure chamber will be maintained as required by experimental conditions. Air flow will be monitored regularly. Oxygen concentration in the exposure chamber will be at least 19% and carbon dioxide will not exceed 1%.

No diet or drinking water will be available during exposure.

Cross and longitudinal sections of the chamber are shown in *Figure 1*:

Longitudinal section	Cross section
	

7 TEST ITEM AEROSOL GENERATION

7.1 Preparation of test item

Whenever possible, an aerosol will be generated from the test material as supplied. However, some preparation may be needed to facilitate aerosolisation, e.g. grinding or sieving of powders. If performed, details of these processes will be included in the final report.

7.2 Generation of the test item atmosphere

The generating system will vary depending on the physical characteristics of the test item. Details of the generation system used will be documented in the raw data and given in the final report.

To obtain the target concentration levels, the aerosol will be diluted with filtered air from a compressor and conveyed via glass tubing from the generator to the exposure chamber. The flow rate through the exposure chamber will be adjusted as necessary.

Previous to exposure onset, a complete setup of the system and characterisation of test atmosphere will be performed according to the corresponding SOPs. The tasks will be the following:

- Making an outline of each line including identification of the corresponding equipment.
- Leak test through the aerosol conveyor glass tubes.
- Identification of each group (name, dose, total number of animals, total flow of aerosol/air).
- Checking of air temperature and relative humidity.
- Theoretical calculations regarding dilution and Venturi tubes to be used.
- Vacuum system checking in sampling ports: capillary installed, vacuum pressure measurement, sampling flow.
- Exposure test: determination of pressure inside the inhalation chamber versus room pressure and difference of pressure between both chamber compartments. Determination of aerosol flow and

aerosol concentration in different positions of the chamber randomly selected: six ports from the high level and six ports from the low level. Calculation of coefficient of variation (%CV) within each level and across the two levels. Within each level, the CV will not be higher than 5%, whereas the CV among the two levels will not exceed 10%.

8 CHARACTERISATION OF TEST ATMOSPHERES

In order to characterise the test atmospheres and to check the reproducibility of aerosols generation and dilution, several analytical parameters will be determined at defined intervals.

8.1 Determination of the nominal aerosol concentration

The test item usage will be determined once per exposure by weighing the amount placed in the generator reservoir before and after each exposure to determine the quantity of test item used. The weight used will then be divided by the total volume of air passed through the chamber system to give the nominal concentration. A comparison of the nominal concentration and the actual concentration will give an indication of the generation efficiency of the system.

8.2 Gravimetric determination of the aerosol concentration

Gravimetric determination of the aerosol concentration will be performed at least twice in a four hour exposure. Additional samples will be collected if considered necessary.

Test aerosol samples will be collected onto a suitable filter using an appropriate filter sampling device. The sampling flow will be similar to the air flow rate per exposure port. The duration of sampling will be sufficient to ensure reliable results. The filters will be weighed before and immediately after sampling using a calibrated balance. The gravimetric aerosol concentration will be calculated from the amount of test item present on the filter and the sample volume.

8.3 Determination of particle size distribution

The particle size distribution will be determined gravimetrically at least twice during each four hour exposure. Additional samples will be performed at the discretion of the study director.

The cumulative particle size distribution of the test aerosol will be determined using a PIXE cascade impactor. The particle size distribution of the test item in the generated aerosol will be measured by gravimetry analyzing the test item deposited on each stage of the cascade impactor.

The mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) will be calculated on the basis of the results from the impactor, using Microsoft Excel® software (Microsoft Corporation, USA).

To allow for exposure of all relevant regions of the respiratory tract, aerosols with MMAD ranging from 1 to 4 µm with a GSD in the range of 1.5 to 3.0 are recommended.

8.4 Determination of the temperature

The temperature in the aerosol will be measured continuously in each group during each exposure using an appropriate device. Ideally the temperature should remain within the range 22 ± 3ºC. The results will be reported at least three times during exposure.

8.5 Determination of relative humidity

The relative humidity (RH) in the aerosol will be measured continuously in each group during each exposure using an appropriate device. The RH should be maintained in the range of 30-70% but under certain circumstances this may be unattainable or not measurable. The results will be reported at least three times during exposure.

8.6 Determination of oxygen and CO_2 concentration

The oxygen concentration of the test atmosphere will be measured during each exposure using an appropriate device. The oxygen and CO_2 concentration will be maintained above 19% and below 1% respectively, during the exposure period. The results will be reported approximately hourly from the start of the inhalation exposure.

8.7 Airflow rate

The exposure airflow rate will be adjusted as appropriate before the start of the exposure using the pressure difference over a Venturi tube. The actual airflow rate will be recorded at least hourly during exposure. Additional measurements will be performed if considered necessary.

9 EXPERIMENTAL DATA

Experimental data concerning animal group distribution, body weight, general clinical signs, and gross necropsy findings will be recorded through Provantis 8.5.2.1TM (Instem LSS Ltd., Staffordshire, United Kingdom) preclinical software.

9.1 In life observation

9.1.1 Mortality and premature sacrifice

The animals will be examined daily for mortality and morbidity. Any clinical signs, discomfort and mortality will be recorded in accordance with the humane endpoints guidance document of the OECD. Monitoring will be adjusted accordingly if the condition of the animal gives cause for concern.

Where the condition of an animal required premature sacrifice, it will be euthanized by pentobarbital overdose and will be considered in the interpretation of the study results in the same way as animals that died on test. Any decision regarding premature sacrifice will be made after consultation with the Study Director (or a nominated person), if possible.

9.1.2 Clinical observations

Clinical observations in response to treatment will be performed on all animals hourly during exposure (only grossly abnormal signs), immediately after finishing exposure and 1h thereafter. Afterwards, clinical observations will be made once daily until the end of the observation period. All animals will be observed for a period of at least 14 days after administration. The duration of the observation period will be extended if considered necessary by the time of onset and severity of toxic effects.

Any visible clinical signs, discomfort and mortality will be recorded. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy (hypoactivity), irregular respiration, sleep, coma and bodyweight loss. Monitoring will be adjusted accordingly when the condition of the animal would give cause for concern.

9.1.3 Body Weight

All animals will be weighed at least once during the acclimatization period, on the day of treatment just before starting the exposure (day 1 of study), on study days 2, 4 and 8 and weekly thereafter. Animals will be also weighed immediately before sacrifice. Additional body weights will be obtained as necessary for animal welfare reasons.

9.2 Terminal investigation

Animals will be sacrificed at the end of the observation period by an over dose of pentobarbital and subjected to a gross necropsy consisting in the examination of the abdominal and thoracic cavities and contents. All gross pathological changes will be recorded, with particular attention to any changes in the respiratory tract.

Any organ with gross lesions will be collected and preserved in fixation medium (neutral-buffered 4 % formaldehyde) for histological evaluation if considered relevant. Animals dying during the study or sacrificed for animal welfare reasons will be also subjected to a gross necropsy.

10 STATISTICAL ANALYSIS

No statistical analysis will be performed.

11 REPORTING

Vivotecnia will issue an electronic audited draft report before the report is finalised.

The principal investigator from the name delegated phase will prepare a phase report containing the details of the methodology used, experimental conditions and results obtained. This phase report will be signed by the principal investigator. The phase report will be included in the final report.

The final report will contain at least:

- GLP certificate of testing facility
- Certificate of analysis of test item
- Certificate of analysis of water, feed, etc.
- Deviations to the study plan, if applicable
- External contributing reports, if applicable

Number of copies	Final report format	Presentation
01	Electronic	PDF by e-mail*

**PDF files are considered to be outside the scope of FDA 21 CFR Part 11.*

12 STORAGE OF RECORDS AND ARCHIVE

Item	Storage conditions	Duration
Documents: General Study plan, Study-specific supplement and amendments Raw data Animal Facility records Test item storage room records Test item preparation records Final Report Samples: Fixed wet tissues (if applicable)	Room Temperature	4 years

Final destination: after the storage period, items/documents will be made available to the sponsor, who will decide whether they will be sent back or further stored in the GLP archive (please note that a fee may be charged).

13 REFERENCES

Code	Title
2004/10/EC	Good Laboratory Practices
RD 1369/2000	Principios de Buenas Prácticas de Laboratorio
OECD 403	Acute Inhalation toxicity
OECD 39	Guidance document on acute inhalation toxicity testing

END OF GENERAL STUDY PLAN

Study Specific Supplement

Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

STUDY INFORMATION

Study code:	B-02523
Title:	Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

SPONSOR CONTACT INFORMATION

Sponsor	Study Monitor
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Directa Plus Spa	Lab4LIFE Srl
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Tel: +39 02 36714401	Tel: +39 05 1323039
e-mail: carolina.volonte@directa-plus.com	e-mail: lorenzo.autore@lab4life.com

ADDITIONAL RELEVANT PERSONNEL INVOLVED IN THE STUDY

Name	Initials	Position
Raquel Gómez	RGR	Animal facility director and Welfare adviser
Verónica Maillo	VMA	Veterinarian
Jesús Illán	JIM	Technician responsible

STUDY SCHEDULE

The study will be conducted according to the following calendar:

Milestone	Date
Study initiation	Signature of Study Specific Supplement
Experimental starting date (dosing group A, starting dose)	25.04.2018
Experimental completion date (assuming one experimental group)	09.05.2018

The above dates are provisional and may be subject to change. The exact dates will be presented in the final report.

TEST ITEM IDENTIFICATION

Test item: PURE G+	
Batch:	P219/2016
Supplier:	Directa Plus S.p.A
Identification code:	Lab4LIFE ID: 180226-01
Physical and chemical properties	
Physical state:	Solid: Powder
Purity / composition:	> 98%
Correction factor:	None
Colour:	Black-grey
Storage conditions:	Room temperature (ca. 23º C) and protected from light
Homogeneity:	Neat substance
Expiry / retest date:	Not defined*
Handling conditions:	During manipulation of material under chemical hood, set the front glass and the fan speed so that the frontal suction hood is between 0.4 and 0.7 m/s. The height of the frontal safety glass must be kept at 30 cm from the bottom, while maintaining the proper frontal speed. Manipulate the material in order to ensure the least possible dispersion of material. Avoid or minimize the presence of air currents near the chemical hood. Provide a 5 minutes break at the end of the nanoparticle manipulation before proceeding with other activities to allow the concentration of aerodispersed particles to be reduced in the hood. During the activity, use personal protective equipment for handling the material (FFP3 mask, gloves). Proceed to cleaning the hood workpiece after any nanomaterial manipulation activity: the damp cloth is sufficiently efficient; equivalent or more efficient systems are equally valid for the purpose of reducing PM and UFP concentrations. During the activity, use personal protective equipment for handling the material itself (FFP3 mask, gloves). Avoid dust dispersion. Provide a good ventilation when handling. Avoid breathing dust. Keep away from sources of ignition. Graphite is an excellent electricity conductor; prevent dusts accumulation where such accumulations may cause short circuits
Other safety relevant observations:	Eye/face protection: Wear eye protection (complying with EN 166), if prevention measures are not sufficient to reduce the risk of eye contact. Hands and body protection: In case of prolonged or repeated contact with the skin, wear protective gloves of rubber or other material adequate to the specific tasks (complying with EN 374), and appropriate clothes. Respiratory protection: If the dusts dispersion cannot be adequately controlled by appropriate engineering controls, wear respiratory protective equipment, such as masks, half-masks or self-contained breathing apparatus (complying with EN 149, 140 or 136).

** According to the information provided by the sponsor, the test item is stable after two years*

Identification, concentration, composition and any other characteristic needed to define the test item were provided by the sponsor. The sponsor is responsible for the data provided or omitted concerning the characterisation of the test item.

Certificate of analysis will be included in the final report.

EXPERIMENTAL GROUPS

Group	Dose(mg/L)	Exposure time (h)	Males ID	Females ID
A	2	4	1 – 3	4 - 6

According to the information provided by the sponsor, the test item should be tested by reproducing real conditions of use as aerosol with a concentration not higher than 2mg/L as per OECD 403 section 30. Furthermore, this target concentration was found to be the highest technically achievable.

AEROSOL ANALYTICS

No aerosol analytics are considered necessary since the test item purity is higher than 85%.

Study Specific Supplement

Acute inhalation toxicity of the test item PURE G+ in Sprague Dawley rats: OECD Nº 403, Traditional Protocol

APPROVAL

Study Director	Dr. Pilar Prieto
	Date: 24.04.2018
Laboratory Director	Dr. Andrés Konig
	Date:
Study Monitor	Lorenzo Autore
	Date: 25/04/18
Sponsor:	Carolina Volonté
	DIRECTA PLUS S.p.A. Via Cavour, 2 22074 LOMAZZO - CO C.F. e P.I. 04783370960
	Date: 24/04/2018

End of study specific supplement

CONFIDENTIAL Page 4 of 4

END OF FINAL REPORT

Farcoderm
TESTED WELLNESS

tested by Farcoderm

IN VITRO PRODUCT SAFETY STUDY
BIOLOGICAL EVALUATION OF A MATERIAL
IN VITRO SKIN IRRITATION: HUMAN SKIN MODEL TEST FOR THE EVALUATION OF SKIN IRRITATION

DIRECTA PLUS SPA

PURE G+

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COMPLIFE GROUP

Capitale Sociale € 100.000 i.v. - Codice Fiscale e Partita Iva IT 03893350961
Sistema di Gestione Qualità Certificato UNI EN ISO 9001:2008 13659

UNI EN ISO 9001:2008 CERTIQUALITY SISTEMA DI GESTIONE QUALITÀ CERTIFICATO
CERTIQUALITY È MEMBRO DELLA FEDERAZIONE CISQ IQNet

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TEST PROCEDURE INFORMATION

Tested product

DIRECTA PLUS SPA

PURE G+

Sample description
Powder of pristine graphene nanoplatelets

Date of test execution
24/02/2016-26/02/2016

Experimental model
STERLAB EPIDERMIS age 17 batch N° 1602 EPID 01
0,5 cm^2 reconstructed epidermis of EP.AJ P1 21. keratinocytes. Cells are grown on inert polycarbonate filter on chemically defined medium, airlifted for 17 days.

Culture medium
MAINTENANCE MEDIUM batch N° 1602 HC 640

Protocol
In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method (OECD 439)

Positive control
Sodium dodecyl sulphate (SDS) at 5%

Negative control
Saline phosphate buffer (PBS)

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SUMMARY

The purpose of the study is the evaluation of skin corrosivity of a material by using the alternative method «In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method» (OECD 439). The salient features of this method are reported below:

- study performed on reconstituted human epidermis STERLAB EPIDERMIS, a three-dimensional human skin model with a functional stratum corneum;
- deposit of 16 mg ± 2 mg of test material on the surface of epidermis for 42 minutes;
- assessment of cell viability by MTT method. Validation of the test by positive control (5% SDS) and negative control (PBS).

In accordance with the results of cell viability obtained after tissue treatment (95.26%) the product

DIRECTA PLUS SPA

PURE G+

is classified as NON IRRITANT

INTRODUCTION

Study aim

The «In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method» (OECD 439) is designed for the prediction of skin irritation of chemicals by measurement of ability to produce a decrease in cell viability, as reflected in the MTT assay, on the reconstructed human epidermis model. The principle of the human skin model assay is based on the hypothesis that irritant chemicals are able to penetrate the stratum corneum by diffusion or erosion and are cytotoxic to the underlying cell layers.

Bibliographic references

This test takes in consideration the following bibliographic references:

SKINETHIC SKIN IRRITATION TEST-42 BIS (SOP): Validation of the Skinethic skin irritation test-42 bis assay for the prediction of acute skin irritation of chemicals, 2009;

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OECD Guidelines for the testing of chemicals, OECD 439: in Vitro Skin Irritation: Reconstructed Human Epidermis Test Method, 2013;

INVITTOX protocol n. 135/138 (SkinEthic™ Skin Irritation Test -42bis/ In vitro EpiDermTM Skin Irritation Test).

Tested product

DIRECTA PLUS SPA

PURE G+

Sample description

Powder of pristine graphene nanoplatelets

MATERIALS AND METHOD

Sample and control preparation

Liquid and viscous test substances:

16 µL ± 0.5 µL of the undiluted test substance.

Solid test substances:

16 mg ± 2 mg of undiluted test substance.

Three tissues per test substance are used.

Control preparation

5% SDS was used as positive control. PBS was used as negative control.

Tissues

RHE set and media are provided by STERLAB.

Together with the tissues are also provided material quality control that are satisfied: normal histology (absence of significative alterations), cell viability (MTT OD > 0.8), barrier function integrity (4.00 < ET50 < 9.00 hrs), absence of bacteria, fungi, HIV, and Hepatitis B, C.

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Procedure for cell viability evaluation

Pre-incubation step

An appropriate number of 24-well plates was filled with 300 µL maintenance culture medium.
Using sterile forceps, tissues were taken off from agarose and the bottom of the insert wass cleaned on sterile absorbent paper to remove eventual remaining agarose pieces.
The tissues were transferred on fresh medium and placed at 37°C, 5% CO_2, 95% RH incubator until test substance application.

Application of test substance and rinsing

24-well plates were filled with 300 µL maintenance culture medium pre-warmed at room temperature:
Using sterile forceps, tissues were transferred in the new plates.
Test was performed in triple: 3 wells per test substance, 3 for positive control and 3 for negative control for each time point.

Topical application: 42 minutes treatment

Liquid and viscous test substances:

16 µL ± 0.5 µL (i.e. 32 mg/cm²) of the undiluted test substance are applied on the top of each epidermis tissue using micropipette.

Solid test substances:

16 mg ± 2 mg (i.e. 32 mg/cm²) of the undiluted substance are applied to the epidermis surface; 10 µL of deionized water are added to ensure a good contact of the sample with the skin surface.

Rinsing and drying steps

Tissues were rinsed with 25 mL PBS to remove all residual test substance from the epidermal surface. Insert bottom was dried on a sterile absorbent paper. The surface of the tissues was dried with sterile cotton tip.
Washed tissues are then transferred into 2 ml of fresh culture medium and maintained for 42 hours in incubator at 37°C, 5% CO_2 for the recovery phase before being processed for MTT assay for the assessment of cell viability.

MTT test

Tissue viability is assessed by MTT reduction measurement, after the 42 hours recovery phase.

Incubation in MTT solution

Dye solution was freshly made at the final concentration of 1 mg/ml in PBS and filtered with 0.22 µm.
24-well plates were filled with 300 µL MTT and incubated for 3 hours (± 5 minutes) at 37°C, 5% CO_2, 95% RH.

Formazan extraction

24-well plates were filled with 800 µL 2-propanol.
At the end of the 3 hours (± 5 minutes) incubation in dye solution, tissues were transferred in 2-propanol.
700 µL 2-propanol were added on the top of each tissue and incubated for 2 hours (± 5 minutes) at room temperature with gentle agitation for formazan extraction.
Plates were parafilmed to avoid solution evaporation.

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Optical density measurements

At the end of the 2 hours (± 5 minutes) incubation in 2-propanol tissues were pierced with a tip and the extraction solution was homogenized by pipetting up and down to complete formazan solubilization.
3 X 200 µL aliquots per well of extraction solution were transferred in 96-well plates and optical density was read at 570 nm.

Acceptance criteria

TEST QUALITY CONTROL

The study is valid if all quality criteria are satisfied.

Negative Control acceptance criteria

Negative control mean OD value of the 3 tissues should be ≥ 1.2 A at 570 nm.
Viability standard deviation value should be is ≤ 18% (Performance Standards ECVAM SIVS, 2007).

Positive Control acceptance criteria

Positive control mean viability, expressed as % of the Negative Control, should be < 40 % and viability standard deviation value should be ≤ 18 % (Performance Standards ECVAM SIVS, 2007).

Test substance acceptance criteria

Viability standard deviation should be is ≤ 18% (Performance Standards ECVAM SIVS, 2007).

Result calculation and data interpretation

Test substance and positive control percentage of relative viability was calculated in respect to negative control.
Tested sample was classified according to the following criteria:

Mean tissue viability is ≤ 50% = IRRITANT;
Mean tissue viability is > 50% = NON IRRITANT.

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RESULTS AND CONCLUSION

ACCEPTANCE CRITERIA

All acceptability criteria are satisfied.

Negative control (NC)
Mean value OD570 = 1.223 St. Dev. = 5.58%

Positive control (PC)
% mean viability = 6.27% St. Dev. = 0.54%

TEST RESULTS

TESTED PRODUCT	% CELL VIABILITY MEAN VALUE ± ST. DEV.	CLASSIFICATION
DIRECTA PLUS SPA **PURE G+**	**95.26% ± 4.05%**	**NON IRRITANT**

San Martino Siccomario – 1st March 2016 (first emission)

San Martino Siccomario – 19th April 2016 (new editing)

Experimenter

Dr. Gioia BIZZARO

Quality control

Dr. Angela MICHELOTTI

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BIBLIOGRAPHY

SKINETHIC SKIN IRRITATION TEST-42 BIS (SOP): Validation of the Skinethic skin irritation test-42 bis assay for the prediction of acute skin irritation of chemicals, 2009.

OECD Guidelines for the testing of chemicals, OECD 439: in Vitro Skin Irritation: Reconstructed Human Epidermis Test Method, 2013.

INVITTOX protocol n. 135/138 (SkinEthic™ Skin Irritation Test -42bis/ In vitro EpiDermTM Skin Irritation Test).

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IN VITRO EVALUATION OF THE ALVEOLAR TOLERABILITY OF A PRODUCT ON RECONSTRUCTED TISSUES

DIRECTA PLUS SPA

PURE G+

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REPORT CHANGE RECORD

The table here below reports the change log of all approved changes made to the document that make up the course after initial approval.

Rev. no	Date	Description
00	12/01/2021	Draft Report Release
00	16/02/2021	Final Report Release

1. STUDY DESIGN

1.1 Title

In vitro evaluation of the alveolar tolerability of a product on reconstructed tissues

1.2 Study aim

Evaluation of alveolar epithelium tolerance of a product by means of the evaluation of the viability of the tissue used in the test and the release of TNF-alpha (proinflammatory acute cytokine) following the treatment with the product under exam.
The salient features of used method are reported below:

- Study performed on reconstituted human alveolar epithelium STERLAB ALVEOLAR EPITHELIUM, a three-dimensional model;
- 300 µl of product were deposited on the surface of the epithelium in a single and prolonged application for 6 and 24 hours in suspension at 100 mg/ml;
- assessment of cell viability by MTT method;
- validation of the test by positive control (5% SDS) and negative control (PBS);
- TNF-alpha dosage by means of ELISA assay

1.3 Tested product

DIRECTA PLUS SPA

PURE G+

Sample description

Graphene nanoplatelets from graphite

1.4 Date of test execution

Experimental execution: 10/12/2020-11/12/2020
Dosage: 23/12/2020

2. MATERIALS AND METHODS

2.1 Sample and control preparation

The sample was tested as it is and in the concentration of 100 mg/ml by means of direct application.
The quantity of product to apply on the tissue surface was chosen according to literature data and product instructions of use.
The product was applied on the entire tissue surface for 6 hours and 24 hours in single and prolonged application
The test was performed in triplicate.

Control preparation

Extemporaneously SDS 5% solution (Positive Control). The % SDS solution must be made in weight / volume (weighing of the SDS then add distilled water until the necessary volume to reach the final concentration of 5 % W/V).
As negative control phosphate saline buffer was used (PBS).

2.2 Tissues and media

Tissue set and media are provided by Sterlab.
The tissues and the necessary culture media (maintenance medium) are subjected to quality control by the provider and recorded on analysis certificate.

STERLAB Alveolar Epithelium batch N° 2011 CALU 01

0.5 cm^2 reconstucted buccal ephitelium reconstituted by airliften culture of TR146 cells for 5 days in chemically defined medium on inert polycarbonate filters.

Description	**Epithelium alvéolaire humain reconstruit** *Reconstructed Human Alveolar Epithelium*		
	Epithélium alvéolaire 0.5cm² reconstitué à partir de la lignée cellulaire Calu3, et cultivé sur du milieu défini à l'interface air/liquide pendant 12 jours. *0,5 cm² alveolar epithelium cultivated for 12 days from Calu3 cell line, reconstituted by airlifted culture on insert polycarbonate filter 0.4µm.*		
N° Lot / *Batch N°*	**2011 CALU 01**		
Origine / *Origin*	CALU P31 4		
Utilisation / *Usage*	UTILISATION UNIQUEMENT SCIENTIFIQUE / *FOR SCIENTIFIC USE ONLY*		
Stockage / *Storage*	Les tissus sont reconstruits et emballés dans les conditions stériles. Mettre dans l'incubateur à 37°C ± 1°C, 5% CO_2, 95 ± 5% humidité. *This product was prepared and packaged using aseptic techniques. Store in an incubator at 37° ± 1°C, 5% CO_2, 95 ± 5% humidity.*		
Histologie (coloration HE) *Histology (HE staining)*			
Epaisseur à 12 jours *Thickness at day 12*	50 µm (valeur indicative / *indicative value*)		
Contrôles Qualité à 12 jours *Quality Controls at day 12*	**Test**	**Specification**	**Résultat / *Result***
	Histologie / *Histology* N° fixation: 20427	Nombre de couches ≥ 2 *Number of cell layers ≥ 2*	2
	Viabilité cellulaire (MTT à 550nm) *Cell Viability at day 12 (MTT read at 550 nm)*	**O.D > 0.7**	O.D = 1,399 (n = 1)
Précaution / *Handling*	Niveau – 2 : Les mesures de précaution adéquates doivent toujours être prises par rapport à ce type cellulaire décrit dans « Biosafety in microbiological and biomedical laboratories », 4th ed. HHS Publication N° (CDC) 93-8395. *Level – 2: Biosafety caution must be taken according to specific cells. To know these biosafety cautions, see : biosafety in microbiological and biomedical laboratories, 4th ed. HHS Publication N° (CDC) 93-8395.*		
Sécurité biologique *Biological safety*	**Mycoplasme / *Mycoplasma***	**Sérologie / *Serology***	
	Négatif / *Negative*	Hépatite C : Négatif / *Negative* Hepatite B: Négatif / *Negative* HIV: Négatif / *Negative*	
Expiration date	15/12/2020 - 7 jours après envoi / *7 days after shipping*		
Conformité / *Conformity*	**Conform**		

MAINTENANCE MEDIUM batch N° 20HC331

2.3 Test procedure for MTT test

Pre-incubation step

Pre-incubation step proceed for at least 2 hours.
An appropriate numbers of 6-well plates are filled with 1000 µL mainteinance culture medium.
Using sterile forceps, tissues are taken off from the agarose, the bottom of the insert is cleaned on sterile absorbent paper or gauze to remove eventual remaining agarose pieces. The tissues are transferred on fresh medium by first sloping the insert before complete insert setting at the air-liquid interface. Tissues are placed at 37°C, 5% CO_2 until test substance application.

Application of test substance and rinsing

The maintenance culture medium is pre-warmed at room temperature.
24-well plate are filled with 300 µL pre-warmed maintenance culture medium. Using sterile forceps, tissues are transferred by first sloping the insert before complete insert setting at the air-liquid interface.

Applications of tested product

The product applied so that to completely cover the entire surface of the tissue for 6 and 24 hours in single and prolonged application.

Rinsing and drying steps

At the end of the treatment by placing a funnel in a large beaker (to avoid underneath projections/contaminations of the tissues) the tissues are rinsed thoroughly several times with 25 mL PBS at a 5-8 cm distance from the insert to remove all residual test substance from the tissue surface. Insert bottom is dried on a sterile absorbent paper or gauze for 1-2 seconds. The surface of the tissue is sweepped with both ends of a cotton tip. Washed tissues are transferred in MTT solution.

2.4 MTT test

Tissue viability is assessed by MTT reduction measurement.

Incubation in MTT solution

A 24-well plate is filled with 300 µL MTT (1 mg/ml).
The treated tissues are transferred in the pre-filled MTT 24-well plates, by first sloping the insert before complete insert setting at the air-liquid interface and incubated for 3 hours (+/- 5 minutes) at 37°C, 5% CO2, 95% humidified atmosphere.

Formazan extraction

A new 24-well plate is filled with 1500 µL isopropanol.
At the end of the 3 hours (± 5 minutes) incubation in MTT solution, treated tissues are transferred in isopropanol solution and incubated for 2 hours (± 5 minutes) at room temperature with gentle agitation (about 150 rpm) for formazan extraction.

Optical density measurements

At the end of the 2 hours (± 5 minutes) tissue and polycarbonate filter are pierced with a tip in order to get the whole extraction solution in the corresponding well.
The extraction solution is homogenized to complete formazan crystals solubilization.
200 µL extraction solution per well are transferred into a 96-well plate and optical density is red at 540 nm.

2.5 Result calculation and data interpretation

% relative viability is calculated for test substance and positive control respect to negative control. Tested sample is classified according to compared to the following criteria:

Mean tissue viability is ≤ 50% Irritant
Mean tissue viability is > 50% Not Irritant

2.6 TNFalpha dosage

Culture media of controls and tissues treated with tested product were used for the dosage of pro-inflammatory cytokine TNFalpha by means of ELISA method.
Commercial kits were used for the determination. ELISA use the competitive binding between an antigen (in this case the cytokine of interest) and its primary antibody. The immune complex (antigen-antibody) was bond by a secondary antibody conjugated to a peroxidase. The addition of the enzyme substrate gives a colorimetric reaction with intensity proportional to the immune complex presence, and so to the cytokine quantity.
The quantitative determination uses a calibration curve made-up of standard known and growing concentrations of standard cytokine.

2.6 Bibliographic references

OECD Test No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method

OECD Test No. 492: Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage

ISO 10993-1:2009 Biological evaluation of medical devices -- Part 1: Evaluation and testing within a risk management process

ISO 10993-2:2006 Biological evaluation of medical devices -- Part 2: Animal welfare requirements

ISO 10993-10:2010 Biological evaluation of medical devices -- Part 10: Tests for irritation and skin sensitization

ISO 10993-12:2012 Biological evaluation of medical devices -- Part 12: Sample preparation and reference materials

In Vitro EpiDerm™ Skin Irritation Test (EPI-200-SIT)

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Nielsen HM, Rassing MR. TR146 cells grown on filters as a model of human buccal epithelium: IV. Permeability of water, mannitol, testosterone and beta-adrenoceptor antagonists. Comparison to human, monkey and porcine buccal mucosa. Int J Pharm. 2000 Jan 25;194(2):155-67.

3. RESULTS

3.1 Cell viability-MTT test

Table 1 – % cell viability in tissues treated with test control and tested product for 6 or 24 hours. The results are expressed as mean % variation ± dev.st. compared to the negative control.

TEST CONTROL	% CELL VIABILITY (MEAN VALUE ± ST. DEV.)	CLASSIFICATION
SDS 5%	6 h exposure **9.45% ± 0.10%**	**IRRITANT**
	24 h exposure **9.87% ± 0.30%**	**IRRITANT**

TESTED PRODUCT	% CELL VIABILITY (MEAN VALUE ± ST. DEV.)	CLASSIFICATION
DIRECTA PLUS SPA PURE G+	6 h exposure **99.46% ± 1.02%**	**NOT IRRITANT**
	24 h exposure **91.67% ± 5.02%**	**NOT IRRITANT**

3.2 Cytokine release – TNFalpha dosage

Table 2 – TNFalpha dosage on culture media of CTR-, CTR+ and tissues treated with test sample for 6 or 24 hours. The results are expressed as mean value ± dev.st. (expressed in pg/ml) and as mean % variation compared to the negative control. Obtained results were subjected to statistical analysis by means of Student test. Variations (vs CTR-) are considered statistically significant with *p<0.05.

Exposure time: 6h - TNFalpha Dosage (pg/ml)

	MEAN	DS	
CTR-	88,00	29,86	
CTR+	225,50	18,03	*
PURE G+	122,17	17,56	

Exposure time: 24h - TNFalpha Dosage (pg/ml)

	MEAN	DS	
CTR-	90,50	24,49	
CTR+	263,83	30,55	*
PURE G+	117,17	11,55	

The assay of the pro-inflammatory cytokine TNFalpha shows that a statistically significant inflammatory reactivity was induced in the positive control (p <0.05 vs CTR-).
Tissues treated with PURE G + did not show statistically significant changes in the release of TNFalpha compared to CTR- (p> 0.05 vs CTR-) and therefore no inflammatory reactivity was highlighted.

4. CONCLUSIONS

According to the data obtained and reported in this report and according to the applied experimental protocol

DIRECTA PLUS SPA

PURE G+

doesn't show an irritation potential on reconstructed alveolar epithelium

Experimenter	*Study Director*
Dr. Manuela Lanzafame	**Dr. Silvana GIARDINA**
Digitally signed by Manuela Lanzafame Date: 2021.02.16 17:00:14 +01'00'	Digitally signed by Silvana Giardina Date: 2021.02.16 17:08:18 +01'00'

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IN VITRO EVALUATION OF THE ABSORPTION POTENTIAL OF A PRODUCT THROUGHT RECONSTRUCTED HUMAN EPIDERMIS

DIRECTA PLUS SPA

Pure G+

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REPORT CHANGE RECORD

The table here below reports the change log of all approved changes made to the document after initial approval.

Rev. no	Date	Description
00	12/01/2021	Draft report release
00	17/02/2021	Final report release

1. STUDY DESIGN

1.1 Title

In vitro evaluation of the penetration skin absorption of a product through reconstructed human epidermis.

1.2 Study aim

The purpose of the test was the evaluation of the skin absorption potential of the graphene Pure G+ through an in vitro reconstructed human epidermis.
The penetration study was performed following the general principles of OECD 428.

The biological model used in the test was

EpiDerm™ by Mattek

Certificate of Analysis

MatTek | IN VITRO LIFE SCIENCE LABORATORIES

Product: EpiDerm™ Reconstructed Human Epidermis

Lot Number: 34108

Part#: EPI-200, EPI-212, EPI-218

Description: Reconstructed human epidermis tissue containing normal human keratinocytes. This product is for research use only. Not for use in animals, humans or diagnostic purposes.

I. Cell source

All cells used to produce EpiDerm™ are purchased or derived from tissue obtained by MatTek Corporation from accredited institutions. In all cases, consent was obtained by these institutions from the donor or the donor's legal next of kin, for use of the tissues or derivatives of the tissue for research purposes.

Keratinocyte Strain: 00267

II. Analysis for potential biological contaminants

The cells used to produce EpiDerm™ tissue are screened for potential biological contaminants. Tests for each potential biological contaminant listed below were performed according to the test method given. Results of "Not detected" indicate that testing for the potential biological contaminant was not observed as determined by the stated test method.

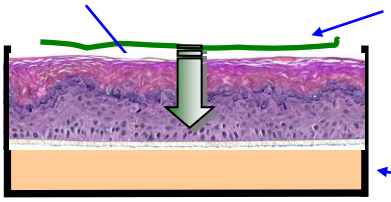
HIV-1 virus – Oligonucleotide-directed amplification	Not detected
Hepatitis B virus – Oligonucleotide- directed amplification	Not detected
Hepatitis C virus – Oligonucleotide- directed amplification	Not detected
Bacteria, yeast, and other fungi – long term antibiotic, antimycotic free culture	Not detected

III. Analysis for tissue functionality and quality

Test	Specification	Acceptance criteria	Result and QA Statement	
Tissue viability	MTT QC assay, 4 hours, n=3	OD (540-570 nm) [1.0-3.0]	1.986 ± 0.018	Pass
Barrier function	ET-50 assay, 100 µL 1% Triton X-100, 4 time-points, n=3, MTT assay	ET-50 [4.77-8.72 hrs]	6.17 hrs	Pass
Sterility	Long term antibiotic and antimycotic free culture	No contamination	Sterile	Pass

The experimental system is represented in the following image:

Penetration through the epidermis *Product spread on the surface of the epidermis*



Reconstructed human epidermis

Medium for the collection of the penetrated substance/product

1.3 Tested product

DIRECTA PLUS SPA
Pure G+

Sample description

Graphene nanoplatelets from graphite

1.4 Dates

10/12/2020 – 11/12/2020

2. MATERIALS AND METHODS

2.1 Preparation of the test item

The sample was tested as a 100 mg/ml suspension solution by means of direct application.
300 µl of product (corresponding to 30 mg) were applied on tissue surface for 6 and 24 hours. The test was performed in triplicate.

2.2 Exposure method

Pre-incubation step

Pre-incubation step proceed for at least 2 hours.
An appropriate number of 24-well plates are filled with 700 µL mainteinance culture medium.
Using sterile forceps, tissues are taken off from the agarose, the bottom of the insert is cleaned on sterile absorbent paper or gauze to remove eventual remaining agarose pieces. The tissues are transferred on fresh medium by first sloping the insert before complete insert setting at the air-liquid interface. Tissues are placed at 37°C, 5% CO_2 until test substance application.

Application of test substance and rinsing

The maintenance culture media is pre-warmed at room temperature.
Wells are filled with 500 µL pre-warmed maintenance culture medium. Using sterile forceps, tissues are transferred by first sloping the insert before complete insert setting at the air-liquid interface.
300 µl of tested product were applied on the surface of the tissue for 6 and 24 hours exposure in single application according to sample posology.
At the end of the treatment by perform the chemical analysis, media and tissues are collected.
The tissues are rinsed thoroughly several times with 1mL PBS at a 5-8 cm distance from the insert to remove all residual test substance from the tissue surface. Insert bottom is dried on a sterile absorbent paper or gauze for 1-2 seconds. The surface of the tissue is swapped with both ends of a cotton tip (5-6 turns per end).

2.3 Dosage

At the end of treatment, the medias underlying the tissues and the tissues were collected.
The dosages were performed on the media and tissues to quantify the amount of absorbed graphene.
The dosages were performed on washing solution too in order to calculate the product recovery (in reference to the applied graphene quantity, as obtained with the same dosage method).
The assays were performed by thermo-gravimetric technique (Weighing after liquid evaporation, 110°C for 4 hours).
For complete data, fresh samples (media and tissue) were measured too.

2.4 Results

On the basis of the obtained experimental data and statistical analysis (T-test), the product penetration is determined. If product penetration is confirmed, the calculation of the quantity of graphene penetrated though the epidermis respect to the quantity of applied product (30 mg) is performed and expressed as a percentage.

3. RESULTS

The results of the test are reported below:

In vitro skin absorption test - Pure G+

Dosages by thermo-gravimetric method

	FRESH MEDIA					
	Weight (g)			mean value	st. dev.	T.test
Untreated epidermis	0,4952	0,4879	0,4923	0,4918	0,0037	
Epidermis treated with Pure G+ - 6h	0,4842	0,49132	0,4824	0,4860	0,0047	0,335567
Epidermis treated with Pure G+ - 24h	0,4963	0,49592	0,4796	0,4906	0,0095	0,86276

	DRYED MEDIA					
	Weight (g)			mean value	st. dev.	T.test
Untreated epidermis	0,0002	0,0004	0,0003	0,0003	0,0001	
Epidermis treated with Pure G+ - 6h	0,0002	0,0001	0,0003	0,0002	0,0001	0,42265
Epidermis treated with Pure G+ - 24h	0,0002	0,0004	0,0002	0,0003	0,0001	0,42265

	TISSUE					
	Weight (g)			mean value	st. dev.	T.test
Untreated epidermis	0,6274	0,6283	0,6275	0,6277	0,0005	
Epidermis treated with Pure G+ - 6h	0,6283	0,6272	0,6315	0,6290	0,0022	0,483173
Epidermis treated with Pure G+ - 24h	0,6303	0,6282	0,6291	0,6292	0,0011	0,233361

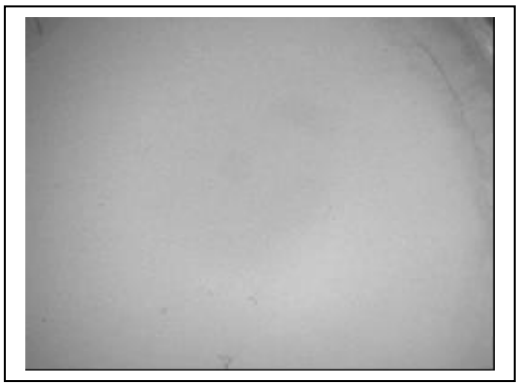
	DRYED WASHING SOLUTION (25 ml)					
	Weight (g)			mean value	st. dev.	T.test
Pure G+ stock suspension (300 ul)*	0,0296	0,0297	0,0296	0,0296	0,0001	
Epidermis treated with Pure G+ - 6h	0,0298	0,0296	0,0296	0,0297	0,0001	0,741801
Epidermis treated with Pure G+ - 24h	0,0296	0,0298	0,0298	0,0297	0,0001	0,225403

* reference value to establish the recovery in the washing procedure

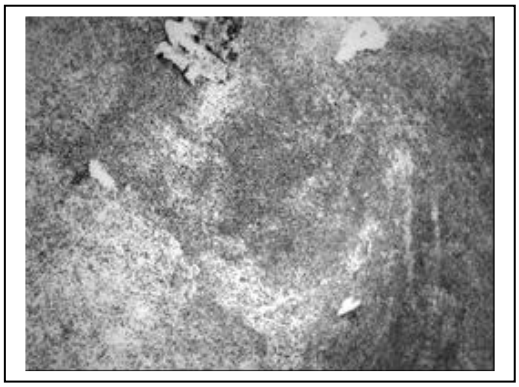
Collected experimental data does not show any absorption potential for G+; no statistical differences were recorded among the control conditions and treated ones, as well as in the washing solution respect to the applied stock suspension.
The product recovery is compliant to 100±10% fixed by OECD 428.

However, some photographic sequences are shown below in which a significant adhesiveness of the product to the surface of the fabric is highlighted, which is not however recorded in quantitative terms through the dosage method used.

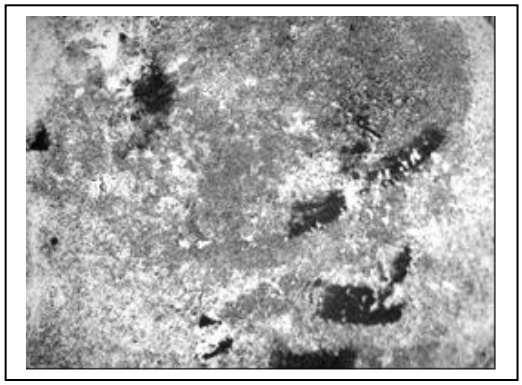
UNTREATED EPIDERMIS



PURE G+ TREATED EPIDERMIS – 6h



PURE G+ TREATED EPIDERMIS – 24h



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Engineered nanomaterials exposure in the production of graphene

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Engineered nanomaterials exposure in the production of graphene

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ABSTRACT

The objective of this study was to obtain the multi-metric occupational exposure assessment to graphene family nanomaterials (GFNs) particles of workers engaged in the large-scale production of graphene. The study design consisted of the combination of (i) direct-reading instruments, used to evaluate the total particle number concentrations relative to the background concentration (time series with spatial approach) and the mean size-dependent characteristics of particles (mean diameter and surface-area concentration) and (ii) filter-based air sampling for the determination of size-resolved particle mass concentrations. The data obtained from direct reading measurement were then used to estimate the 8-h time weighted average (8-h TWA) exposure to GFNs particles for workers involved in different working tasks. Workers were generally exposed to 8-h TWA GFNs particle levels lower than the proposed reference value (40,000 particle/cm^3). Furthermore, despite high short-term exposure conditions were present during specific operations of the production process, the possibility of significant exposure peaks is not likely to be expected. The estimated 8-h TWA concentration showed differences between the unexposed ($<$100 particle/cm^3; $<$0.05 μg/m^3) and exposed subjects (mean concentration ranging from 909 to 6438 particle/cm^3 and from 0.38 to 3.86 μg/m^3). The research outcomes can be of particular interest because the exposure of workers in real working conditions was assessed with a multi-metric approach; in this regard, the study suggests that workers who are directly involved in some specific working task (material sampling for quality control) have higher potential for occupational exposure than operators who are in charge of routine production work.

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Introduction

Background

The graphene family of nanomaterials (GFNs) has been recently introduced into many fields of science and technology (Jang and Zhamu 2008; Rafiee et al. 2009). Given the potential occupational and public exposure to graphene due to its versatile applications, scientists are directing more attention toward investigating the safety aspects of these nanomaterials (Hu and Zhou 2013). What is emerging from the available results is a variety of effects that are strictly related to the nature of the graphene used: the size, layer number, chemical groups, and surface of graphene may have a strong impact on the biological and toxicological responses (Hu and Zhou 2013). Nevertheless, to date, no occupational or environmental exposure limits for GFNs have been set by any regulatory agency (Lo et al. 2011), and limited data are available regarding occupational exposure assessment in the GFNs production industry. In summary, further research is still required in this emerging field to draw conclusions regarding the potential hazards and risk characterization by way of the link between these exposure assessments (Allen et al. 2010; Guo et al. 2010; De et al. 2011; Bianco et al. 2013).

The preliminary hypothesis of this study was that the production process may cause occupational exposure arising from the emission and dispersion of GFNs particles, mainly in the form of airborne nanoparticles ("NPs," i.e., particle with diameter $<$100 nm). The main objective of the study is to assess the occupational exposures to GFNs of workers engaged in the large-scale production of graphene. The exposure assessment was performed via environmental monitoring and aimed at the multi-metric characterization (i.e., particle number, mass and surface-area concentrations, particle mean

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diameter) of the NPs exposure concentration associated with each working task and working place.

The production process

The production process in the study company is a continuous manufacturing process based on the chemical intercalation of natural graphite, followed by thermal plasma expansion. The production unit (capacity of 30 tonnes per year) was designed with a modular pattern and to be fully automated with minimal human intervention; only quality-control material sampling, cleaning, and maintenance operations required manual interventions. The production was organized as follows: (i) acceptance of raw materials (graphite) and storage; (ii) plasma expansion; (iii) post-plasma treatment/exfoliation (performed in liquid media); (iv) drying; (v) finishing operations (e.g., packaging), and (vi) storage of final products. Quality controls of raw materials, by-products, and final products were performed through the entire production process and required material sampling from different points of the production process. All of these tasks were performed within a single building, organized to accommodate separated areas for each specific activity: warehouses for raw materials and finished products were placed in a separate area (downstairs); the production site (upstairs) was divided into a productive area designed with a modular pattern, situated in three containment chambers ("graphite expansion," "exfoliation," and "drying" room) and an open-space area including both R&D laboratory (materials characterization, quality control and testing) and "engineers" workstations (production process monitoring). Finally, the administration department (offices) was located in a separate, adjacent building.

Risk management strategy

It is globally recognized that a general hierarchical approach in risk management must be implemented to eliminate the hazard when possible, substitute it with a less hazardous material or, if not feasible, control the hazard at or as close to the source as possible. The effectiveness of the exposure controls and measurement methods, especially for engineered nanomaterials (ENMs), remains a key research need (Kuempel et al. 2012). In the absence of regulatory occupational exposure limits (OELs) for most of the existing ENMs, a strategy is required to determine the appropriate levels of exposure controls to protect workers' health (Pietroiusti and Magrini 2014). A typical hierarchical risk management approach (Table 1) was implemented at the study company coherently with a precautionary approach, to control the exposure to NPs associated with the production process (Mirer et al. 2008).

Methods

The exposure assessment was performed during a 12-month period (preliminary observational survey and six monitoring sessions). First, a preliminary assessment involved identifying the potential source(s) of NPs emissions by reviewing the type of process and work practices, as suggested by Methner et al. when conducting nanoparticles exposure assessment studies with tiered approach (Methner et al. 2009). Measurements were then performed in accordance with the sampling strategy described hereafter. The measurement design consists of the combination of: (i) direct-reading instruments used to evaluate the total particle number concentrations (PNC), including that of the background and the mean size-dependent characteristics of particles (mean diameter, surface-area concentration); (ii) filter-based PM sampling used for the determination of size-resolved particle mass concentrations.

Monitoring strategy

The measuring strategy for the exposure assessment consisted of: (i) determination of the "background" particle

Table 1. Risk management options used for different work tasks.

<table>
<tr><th></th><th>Production (expansion, exfoliation, drying)</th><th>Maintenance</th><th>Cleaning</th><th>Laboratory test</th></tr>
<tr><td>Organization, Procedures</td><td colspan="4">Warnings, Training, Specific Operating Procedures (S.O.P.)</td></tr>
<tr><td rowspan="2">Engineering Control</td><td colspan="2">Enclosure of high-exposure potential activity: fixed containment booths: HEPA-filtered, double locked-holding area</td><td rowspan="2">+ HEPA-filtered Vacuum Cleaners</td><td rowspan="2">Chemical Hoods (EN 14175-compliant)</td></tr>
<tr><td colspan="2">Closed-process, fully automated production line; General Ventilation</td></tr>
<tr><td>Personal Protective Equipment</td><td colspan="4">Safety Boots and Gloves; Filtering half-facemask (FFP2- or FFP3-class filter); Nitrile gloves and disposable safety gloves</td></tr>
</table>

Table 2. Environmental monitoring strategy and instruments contextually used for different work tasks and environments.

	Production workers			Office workers (controls)
	Graphite expansion	Drying	R&D Laboratory	Office
Background Concentrations (site-specific)	CPC: Particle number concentration (time-resolved) DSC: Particle number concentration, mean diameter; LDSA (time-resolved)			
Micro-environmental fixed-site monitoring	CPC, OPC, DSC, and DLPI: mass concentration, size-resolved (TWA)	CPC: Particle number concentration (time-resolved) OPC: Particle number concentration, size-resolved (time-resolved) DSC: Particle number concentration, mean diameter; LDSA (time-resolved); PCIS: mass concentration, size-resolved (TWA)		
Personal monitoring	DSC: Particle number concentration, mean diameter; LDSA (time-resolved)			n.a.

CPC = Condensation particle counter; DSC = diffusion size classifier; OPC = optical particle counter; DLPI: dekati low pressure impactor; PCIS = Personal Cascade Impactor Sampler; LDSA: lung deposited surface area concentration.

number concentration (natural and anthropogenic nanoparticles in the workplace air); (ii) determination of the total particle number concentration during the production process by means of micro-environmental and personal measurement; (iii) distinction of process-related GFNs particles from background aerosols; (iv) estimation of 8-h Time Weighted Average (8-h TWA) exposures for GFNs particles; and (v) comparison of these exposure values with the available occupational exposure thresholds.

Table 2 summarizes the monitoring design and strategy: average airborne PNC was measured in each location (workplace micro-environments) before and after the production or handling of nanomaterial to obtain an average background number concentration, which is then subtracted from the measurements made during processing, manufacturing, or the handling of ENMs (assuming that the emissions during process are stable during the measurements) (Brouwer et al. 2009; Methner et al. 2009, Koivisto et al. 2012a,b; 2014; 2015; Koponen et al. 2015; Jensen et al. 2015). This approach basically assumes that the concentration determined in each location, during no work activity is representative for the background concentration and any increased concentrations during the work activity can be attributed to the process, the nanomaterial or both (Kuhlbusch et al. 2011).

Once the background particle number concentrations have been determined, specific measurements are made with all of the available instruments simultaneously at different locations: the results from this type of measurement should be interpreted as an indicator of workplace's micro-environment exposure concentration. Exposure to GFNs particles was estimated by comparing the workplace concentrations with the background concentration, following different approaches for background distinction (Kuhlbusch et al. 2011; Berges et al. 2013). However, note that these approaches are considered as a proxy for assessing NPs exposures, despite there being several possible errors associated with use of the count-difference methods for background distinction. In this study, a precautionary and conservative approach was adopted, attributing the whole differential particle concentrations as GFNs particles (without any differentiation between incidental and process-related engineered nanoparticles).

The sampling time generally matched the length of time necessary to complete an entire work shift or a half work shift. Direct reading instruments were placed at both the process/task location (where operations involved engineered nanomaterial production or application, i.e., graphite expansion, drying, R&D laboratory) and at the process/task location (where process nanomaterials were not directly involved [office]). The exfoliation room was excluded from environmental monitoring because workers were not required to work for extended periods in this location and, the exfoliation phase was conducted in closed-process conditions, for which GFNs particles emissions in the workplace were excluded. A time-activity diary was also used to separate the continuous data as a function of the different monitored environments and working tasks. Filter-based, size-selective air sampling ("DLPI": Dekati® Low Pressure Impactor) was also performed in a selected location (graphite expansion, drying) following a worst-case exposure scenario approach (highest expected exposure concentrations). Finally, personal sampling was performed using the miniature diffusion size classifier. Personal measurements were collected in the breathing zone of workers for whom exposure can be expected (e.g., during graphite expansion and drying phases). All of the instruments were used simultaneously for the entire length of the

monitoring period, except for the DSC (which was alternatively used for personal sampling and fixed-site monitoring) and for the personal cascade impactor sampler (PCIS), which were used for extemporary measurements in one monitoring session.

Direct-reading measurements

Numerical concentrations of airborne particles were measured using a miniature diffusion size classifier (DSC) and condensation particle counters (CPC). The DSC used for this study (DiSCmini, Matter Aerosol AG, Wohlen AG, Swiss) measures the particle number concentrations (range: 10^3–10^6 particle/cm^3) and particles' average diameter in the size range of approximately 10–700 nm (Fierz et al. 2011). DSC also estimates with reasonable accuracy (Fierz et al. 2011) the lung deposited surface area concentration (LDSA), defined as the particle surface area concentration per unit volume of air, weighted by the deposition probability in the lung and calculated according to ICRP report 66 (ICRP 1994). Portable CPCs were also used in this study (P-Trak Ultrafine Particle Counter model 8525; TSI Inc., Shoreview, MN, USA) to quantify the airborne particle number concentration(size range: 0.02 to 1 μm). Finally, numeric concentrations of airborne particles were also measured using optical particle counters ("OPC," mod. Handheld 3016, Lighthouse Worldwide Solutions, Fremont, CA, USA), which are able to provide real-time measurement of particles with optical diameters in the 0.3–30 μm range (six different dimensional fractions).

Filter-based PM sampling and gravimetric analysis

The gravimetric determination of the airborne size-fractionated airborne particulate matter (PM) was conducted to characterize the mean exposure to size-segregated PM in terms of mass concentrations (μg/m^3). A DLPI Low Pressure Impactor was used: DLPI classifies airborne particles into 13 size fractions (from 30 nm to 10 μm) at a sampling flow rate of 30 l/min. Sampling were conducted by means of greased filters (aluminium membranes, 25-mm diameter, greased with Apiezon-L); a continuous control of impactor's pressure (100 $\pm$ 5 mbar) was performed during the sampling period to ensure the exact diameter cut point over the measurement period, other than to ensure the accuracy of the sampling volume estimates. A PCIS, which was developed for the analysis of size-segregated particulate matter, was also used for extemporary measurements in one monitoring session. PCIS is a miniaturized cascade impactor, which operates at a flow rate of 9 l/min and consists of four impaction stages with cut-off diameters of 2.5, 1.0, 0.5, and 0.25 μm (PTFE s/PTFE filters; diameter: 25 mm, porosity: 0.8 mm), which are followed by an after-filter for particles <0.25 μm (PTFE w/PMP ring; diameter: 37 mm; porosity: 2 μm) (Misra et al. 2002). Size-resolved mass concentrations (μg/m^3) were then determined by gravimetric analysis in accordance with reference methods and with the accepted standard practice (UNI EN 12341:1999; UNI EN 14907:2005). The net PM mass on the filters was measured by weighing the conditioned filters before and after sampling with a microbalance having a resolution of 1 μg in a temperature- and relative humidity-controlled (20 $\pm$ 1°C; 50 $\pm$ 5%) environment (Activa Climatic; Aquaria, Lacchiarella, MI, Italy). PM masses were corrected by subtracting the mean blank weights (two field blank and two laboratory blank) from the sample weights. The quality of the weighing procedure was assessed using the American Society of Testing and Materials (ASTM) D 6552 method (ASTM 2000).

Data treatment and statistical analysis

The GFNs particles' numeric concentrations (obtained after background concentration subtraction), mean diameter, and LDSA were used to estimate the 8-h TWA exposure to graphene NPs for workers involved in different working tasks, by means of a microenvironmental model. The basic concept of this type of model is that time-weighted average exposure is defined as the sum of partial microenvironmental exposures, which are determined by the product of the GFNs particles concentration and the time spent in each microenvironment/work task. For this purpose, time-activity patterns were derived from time-activity diaries and a simple questionnaire submitted to workers. Workers were *a priori* classified as "non-exposed" ($N = 5$; office workers) or "exposed," including two groups of production workers: "operators" ($N = 3$; assumed to be exposed and directly involved in the production process) and "engineers" ($N = 6$; assumed to be exposed but not directly involved in the production process). A further estimation was performed to obtain the 8-h TWA exposure values expressed as mass concentrations (μg/m^3). The calculation was performed on the basis of a simple algorithm already applied in previous studies on ultrafine particles (Wittmaack et al. 2002; Spinazzè et al. 2015), based on the relationship between the mean mass density factors of NPs ($\rho = 0.05$ g/cm^3) derived from the gravimetric determination of $PM_{0.1}$ in the expansion room, mean particle volume (derived from mean particle diameter), and 8-h TWA exposure expressed as PNC. Note that this technique presents some limitations (Spinazzè et al. 2015) thus, the following results are not expected to provide extremely accurate exposure values but are indicative of the magnitude of the exposure.

The use of non-parametric methods was considered the most appropriate because the data results are skewed. Differences in the median concentrations as a function of working conditions/tasks were tested with the Kruskal-Wallis, one-way ANOVA, and the "MW": Mann-Whitney *U*-test performed using an IBM SPSS 20.0 (IBM, Armonk, NY, USA). Statistical results were regarded as a significant when $p < 0.05$. The results are presented in the text as the geometric mean (GM) ± geometric standard deviation (GSD).

Results

Overall, six monitoring campaigns were performed, after an initial assessment survey, for a total of over 50 of field survey. During the initial survey, direct-reading instruments were used to obtain a semiquantitative indication of the magnitude of potential exposures for each process or work task.

The variation of airborne size-fractionated particle concentrations from background concentrations (Table S1, online supplemental information [SI]) is reported in Figure 1.

The results reported in Figure 1 showed that the airborne particle concentrations during the production phase were consistently higher than the corresponding background levels for the sampling locations in the productive area. A high particle number concentration for particles <0.1 μm, combined with a high particle count in the small size range (0.3–0.5 μm), indicates the possible presence of nanoscale particles (expansion room and drying room); conversely, a low count for particles <0.1 μm, in combination with a high count in the larger size range (>1.0 μm) would indicate the presence of large particles and/or agglomerates (Methner et al. 2009). The massive presence of nanometric and submicrometer particles during the production activity (graphite expansion room) was also observed in the expansion area through the gravimetric determination of size-fractionated PM performed by the DLPI sampler (Figure 2). In particular, the mean concentration (GM ± GSD; % cumulative) of PM_1 (97.2 ± 13.0 $\mu g/m^3$; 85.8%), $PM_{0.5}$ (88.8 ± 9.8 $\mu g/m^3$; 77.6%), $PM_{0.25}$ (16.5 ± 4.4 $\mu g/m^3$; 12.0%), and $PM_{0.1}$ (1.4 ± 0.3 $\mu g/m^3$; 1.0%) clearly reflect the high amount of sub-micrometer particle in the work environment, especially compared with spot measurement of size-fractionated particles ($PM_{0,25}$ = 4.3 $\mu g/m^3$; $PM_{0.5}$ = 6.7 $\mu g/m^3$; PM_1 = 9.3 $\mu g/m^3$) performed in other non-productive areas using a PCIS.

Table 3 reports the multi-metric characterization of exposure concentrations to GFNs particles (personal and microenvironmental monitoring). The background number concentrations defined for each location were subtracted from the measurements made during processing, manufacturing, or handling of ENMs; thus, results from this type of measurement were interpreted as the workplace concentrations of Graphene NPs.

The highest mean exposure concentrations were observed in the graphite expansion room, which were one order of magnitude higher than those found in the R&D laboratory and in the drying room. As expected, the mean particle diameters had an inverse relationship with the PNC, while the LDSA had a direct relationship

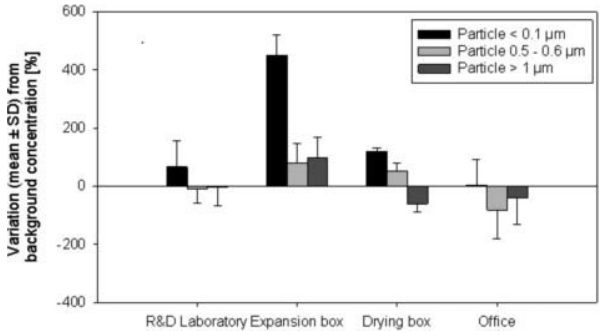

Figure 1. Percent variation of airborne size fractionated particles number concentrations from background concentrations (i.e., particle number concentration measured at a specific location, minus the average background concentration—assumed to be constant—in that specific location; this difference was then divided by the background concentration to obtain the percentual variation from the background concentration) at different sampling locations. Histograms and error bars denote the geometric mean ± geometric standard deviation of the percentual variation. Size fractionated particles number concentrations were measured by means of miniature diffusion size classifier (particle < 0.1 μm) and optical particle counter (particle 0.3–0.5 μm and > 1 μm).

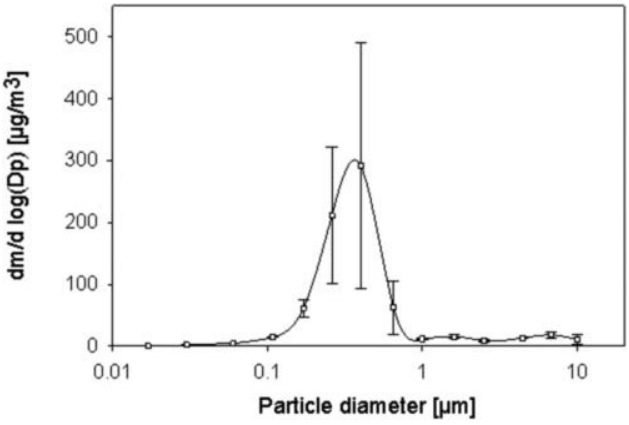

Figure 2. Mass concentrations ($\mu g/m^3$) of size-resolved airborne particles (sampling with Dekati® Low Pression Impactor, and gravimetrical analysis) in the graphite expansion room, during the production activity. Marker with error bars denotes the mean ± standard deviation (concentrations were not corrected for background concentrations).

Table 3. Environmental monitoring results as a function of sampling location, after background concentration subtraction.

Position		GM	GSD	p5	p95
Metric					
R&D Laboratory					
PNC [particle/cm^3]	FS	3779	212	<100	13,521
PNC [particle/cm^3]	P	5882	198	<100	11,927
Particle diameter [nm]	P	91.2	17.8	79.5	104.8
LDSA [μm^2/cm^3]	P	68.02	7.55	65.49	70.51
Graphite expansion room					
PNC [particle/cm^3]	FS	6578	3970	3030	52,200
PNC [particle/cm^3]	P	6050	3676	1862	48,885
Particle diameter [nm]	P	96.8	45.3	37.2	199.4
LDSA [μm^2/cm^3]	P	28.49	25.23	10.57	127.80
Drying room					
PNC [particle/cm^3]	FS	758	553	620	1860
PNC [particle/cm^3]	P	198	230	192	1061
Particle diameter [nm]	P	203.4	68.8	73.2	300.0
Office					
LDSA [μm^2/cm^3]	P	4.41	2.02	3.20	5.91
PNC [particle/cm^3]	FS	<100	73.6	<300	6000
Particle diameter [nm]	FS	136.0	22.9	121.7	150.3
LDSA [μm^2/cm^3]	FS	27.56	5.62	24.58	30.29

FS, fixed-site microenvironmental sampling; P, personal sampling; SD, standard deviation; p(N) = Nth Percentile. PNC: NPs number concentrations [particle/cm^3]; LDSA: lung deposited surface area concentrations [μm^2/cm^3].

with the PNC. Therefore, larger particles and lower LDSA concentrations were primarily found in the drying room, while the smaller particles, with a higher LDSA were measured at graphite expansion room. Statistical analyses were performed to identify significant differences in the multi-metric OEEC characterization as a function of the different working task reported for the graphite expansion and drying activities. All of these work environments were characterized by statistically significant differences ($p_{KW} < 0.001$) in the particle number concentrations (both for fixed-site and personal monitoring), the diameter and the LDSA levels as a function of different work-tasks. In particular, the highest mean concentration and data variability (GM ± GSD) for the graphite expansion process were observed during material-sampling activities (FS: 9677 ± 4719 particle/cm^3; P: 9844 ± 7704 particle/cm/cm^3) and during the following 30-min period (FS: 15243 ± 5297 particle/cm^3; P: 12601 ± 5388 pt/cm^3), rather than during the routine production process (FS: 5643 ± 3987 particle/cm^3; P: 5687 ± 4091 particle/cm^3) or after the end of the production (FS: 4196 ± 323 particle/cm^3; P: 3611 ± 619 particle/cm^3). The same differences were observed for the average particle diameters (which followed an opposite trend compared to the PNC) and LDSA (positive correlation with PNC), showing large average particle sizes and smaller LDSA after the end of the production process (123.3 ± 29.4 nm; 25.93 ± 4.62 μm^2/cm^3) rather than during the routine production process (99.9 ± 40.6 nm; 29.58 ± 19.75 μm^2/cm^3) or during material sampling (53.6 ± 24.5 nm; 30.28 ± 39.43 μm^2/cm^3), and in the 30-min period after that operation (86.6 ± 28.3 nm; 60.18 ± 45.20 μm^2/cm^3). It must be noted that the monitoring of all these operations resulted in the determination of GFNs particles showing smaller average particle sizes and larger LDSA, compared to those determined as background concentrations in the same location (209.3 ± 43.3 nm; 18.42 ± 6.26 μm^2/cm^3). In contrast, smaller but statistically significant differences ($p_{KW} < 0.001$) were observed among the concentrations of exposure concentrations measured during the different work tasks monitored in the drying room, such as cleaning (FS: 942 ± 97 particle/cm^3; P: 554 ± 244 particle/cm^3), graphene-powder packing (FS: 571 ± 98 particle/cm^3; P: 414 ± 190 particle/cm^3), and drying (FS: 765 ± 597 particle/cm^3; P: 110 ± 272 particle/cm^3). Statistically significant differences were also observed for the average particle diameters and LDSA, with larger average particle sizes and smaller LDSA during the drying process (248.8 ± 68.8 nm; 4.48 ± .2.26 μm^2/cm^3), with respect to the background characterization (155 ± 56 nm; 5.8 ± 3.2 μm^2/cm^3), GFNs packing (146.3 ± 65.6 nm; 3.63 ± 2.19 μm^2/cm^3), or cleaning activities (170 ± 63 nm; 5.49 ± 2.71 μm^2/cm^3). In both the graphite expansion room and drying room, each activity-specific multi-metric characterization was significantly different ($p_{MW} < 0.001$) from the others.

Finally, the microenvironmental particle PNC, mean diameter, and LDSA concentrations determined for

Table 4. Time weighted average exposure (TWA 8-h) to graphene NPs for typical workers profile.

Exposure* profile	GM	GSD	p5	p95
Metric				
Not exposed - Office workers (N = 5)				
[Time use: Office = 95.8 ± 5.3%; R&D Laboratory = 4.3 ±5.3%]				
PNC [particle/cm^3]	<100	80	<100	6071
Particle diameter [nm]	134.1	22.7	134.1	150.2
LDSA [μm^2/cm^3]	29.28	5.70	26.42	32.49
Exposed - Engineer (N = 6)				
[Time use: Office = 12.5 ± 30.6%; R&D Laboratory = 87.5 ± 30.6%]				
PNC [particle/cm^3]	3319	195	<100	12396
Particle diameter [nm]	96.8	18.4	96.8	111.5
LDSA [μm^2/cm^3]	62.96	7.31	60.48	65.54
Exposed - Operator (Graphite Expansion) (N = 2)				
[Time use: R&D Laboratory = 5%; Graphite Expansion room = 95%]				
PNC [particle/cm^3]	6438	3782	10659	20113
Particle diameter [nm]	96.6	44.0	46.5	206.4
LDSA [μm^2/cm^3]	30.47	24.35	9.33	125.30
Exposed - Operator (Drying) (N = 1)				
[Time use: R&D Laboratory = 5%; Drying room = 95%]				
PNC [particle/cm^3]	909	536	854	3113
Particle diameter [nm]	197.8	66.3	108.6	364.0
LDSA [μm^2/cm^3]	7.6	2.3	6.43	9.18

*Occupational Exposure to NPs (obtained after background concentration subtraction).

GM, geometric mean; GSD, geometric standard deviation; p(N) = Nth Percentile; PNC: GFNs particle number concentrations [particle/cm^3]; LDSA: lung deposited surface area concentrations [μm^2/cm^3].

different work environments (Table 3) were used to estimate the 8-h weighted exposure to GFNs particles for workers involved in different tasks (Table 4). The 8-h TWA exposure estimation showed differences between the unexposed and exposed subjects; further, exposed operators involved in graphite expansion process are attended to experience the highest exposure level (for to particle with small diameter and wide LDSA), which are one order of magnitude higher than the exposure levels estimated for drying process operator and engineers. Finally, the obtained 8-h TWA exposure levels expressed as mass concentrations showed differences between the unexposed ($<0.05 \pm 0.001$ $\mu g/m^3$) and exposed subjects: for these latter group, in particular, further differences were explored: exposed operators involved in graphite expansion process (3.86 ± 0.085 $\mu g/m^3$) and drying process (3.69 ± 0.078 $\mu g/m^3$) are expected to be exposed at GFNs particle levels one order of magnitude higher than engineers (0.38 ± 0.001 $\mu g/m^3$).

Discussion

The scientific literature about occupational exposure in the production of GFNs is limited: no studies concerning the occupational exposure to nanoparticles reports data on the GFNs production industry (Brouwer et al. 2009; Kaluza et al. 2009; Kuhlbusch et al. 2011; Pietroiusti and Magrini 2014). Making direct comparison with other case studies is quite difficult. Nevertheless, this study shows strong similarities in the exposure characterization to other types of NPs as previous studies showed that (i) under certain circumstances (e.g., maintenance activity, open handling of nanopowders) a release of NPs may occur and that (ii) the pattern of exposure is generally characterized by transient high peaks, linked to specific operations (Magrini and Pietroiusti 2014). In fact, in this study mean particle concentrations (Table 3) were on average of the same order of magnitude as the background level (Table S1, in the SI): by applying the methodology based on the ratio between workplace air concentrations and three times the standard deviation of the background concentration (Asbach et al. 2012a), it may be concluded that nanoparticle emissions during the study process were not significant. Nevertheless, in a specific location (R&D laboratory, graphite expansion room), the peak particle concentrations were one or two orders of magnitude higher than the background levels, and both the standard deviation and 95th percentile were considerably higher than those of the background concentration (Table 3). These are indications of a process in which significantly high short-term exposures may occur. Similarly, the mean exposure concentrations (Table 4) were also in the same order of magnitude (Office workers, Engineers, Drying operators) or one order of magnitude higher (operators working in the graphite expansion room) than background levels (Table S1, in the SI). Further, as mentioned before, the study work environments were characterized by statistically significant differences ($p_{KW} < 0.001$) in the particle number concentrations (both for fixed-site and personal monitoring), the diameter, and the LDSA levels as a function of different work-tasks, confirming that high short-term exposure conditions were present during the production process.

In this regard, a recent technical report performed an evaluation of engineering controls for manufacturing and handling graphene nanoplatelets in the workplace and concluded that some specific tasks in the production process were identified as the sources of release of nanoparticles into the workplace (Lo et al. 2011); the same report also stated that appropriate engineering controls could help mitigate exposure to nanomaterials in production areas. Similarly, in the present study, specific work tasks showed the potential to cause serious contamination within the workplace; however, the development of properly guided procedures for workplace and worker surveillance, together with the improvement of removal and containment systems as well as education and training workers, assisted by periodic exposure assessment activities, were all helpful in preventing workplace contamination and to contain workers' exposure. The definition of specific standard operating procedure was part of a comprehensive risk-management program (Table 1) that also addressed other issues, such as the abatement of air contamination in isolated work areas, the protection of non-productive areas from possible contamination events, and the protection of workers engaged in the production process with adequate personal protective equipment. The implementation of up-to-date control strategies (e.g., improvement of the existing local exhaust ventilation systems with mobile HEPA-filtered inlets to be placed in correspondence with the potential sources of NPs) is recommended and is now under consideration: this should contribute to a lowering of occupational exposures, workplace contamination levels, and high-level transient exposure peaks.

It also must be considered that, to date, no legal binding framework concerning ENMs-specific OELs exist, but several unofficial OELs for ENMs are being proposed by national organizations. In particular, no specific regulatory OEL for graphene NPs were defined (Lo et al. 2011; Pietroiusti and Magrini 2014). In the absence of regulatory OELs, alternative strategies are required for risk assessment purposes, e.g., the determination of technical exposure thresholds for the protection of workers' health. In this regard, exposure levels can be compared with reference values (RVs), developed to provide provisional limit values in situations where recognized OELs and DNELs are not available. RVs represent a warning level; when they are

exceeded, exposure control measures should be taken. In this case, the nanoparticles were bio-persistent, granular, and fiber-form in the size range of 1–100 nm with a density of <6.0 g/cm^3. Therefore, an 8-h TWA RV of 40,000 particles/cm^3 may be adopted as RV (Cornelissen et al., 2012; Van Broekhuizen et al., 2012; Pietroiusti and Magrini, 2014). Note that the adopted RV, as the vast majority of the proposed OELs, is based on the number concentration as reference metric (although some of the proposed OELs are also expressed as mass concentration). Until reliable, easy-to-use and inexpensive devices are developed, this metric should remain the reference to allow for data comparability. Other metrics, such as surface-area concentration, still has limited use, and, overall, no proposed OELs have been developed in relation to this metric (Pietroiusti and Magrini 2014). According to the performed measurements and estimations, workers were exposed to GFNs particle levels (8-h TWA) substantially lower than adopted RV; it should be considered, however, that the most probable exposure in the workplaces is represented by transient spikes occurring during some workplace procedures (Pietroiusti and Magrini 2014). Taking this characteristic into account, the American Conference of Governmental Industrial Hygienists established that a nanotechnology process could be considered to require further assessment if (i) short-term exposures exceed three times the RV for more than a total of 30 min per 8-h working day or (ii) a single short-term value exceeds the RV by five times (ACGIH 2010). None of these two conditions apply here because (i) concentrations above three times the adopted RV (120,000 particle/cm^3) were achieved only for a single short-term episode (<1 min) (ii) no single value exceeded by five times the RV (200,000 particle/cm^3).

Some limitations in the study design and methods could have had an impact on the exposure assessment, including possible errors associated with use of the count-difference method to estimate the particle number concentrations of GFNs particles and direct-reading instruments sensitivity. In particular, the distinction of GFNs particles from background aerosols was performed with a conservative approach, by the quantification of the pre-and post-activity concentrations (background) and assuming that the emissions during process are stable during the measurements (Koivisto et al. 2012a,b, 2014, 2015; Koponen et al. 2015; Jensen et al. 2015). This approach attributes the whole background-differential concentrations to GFNs particles without any differentiation between incidental and process-related nanoparticles; thus, it is plausible that sources of incidental nanomaterials, and the large fluctuations that may result from their presence, may make it impossible to identify actual releases of ENMs and, in conclusion, this approach was likely to result in an overestimation of graphene NPs exposures, mainly for non-productive areas where the presence of graphene NPs is not expected.

Further, at present, the available measurement techniques generally present some limitations in terms of specificity and selectivity, and therefore every attempt to assess the occupational exposure to NPs requires the use of multiple sampling and monitoring techniques. However, these techniques are not yet fully evaluated in terms of accuracy and reliability (in particular those measuring number concentration and surface-area) and are not portable (i.e., multistage impactors for the determination of mass concentrations) so that exposure characterization is deeply limited by the lack of availability of instrumentation for collecting high-quality data, especially for personal monitoring. In this regard nano-specific monitors and samplers are subject to large studies (Kaluza et al. 2009; Asbach et al. 2012b; Kaminski et al. 2013; Price et al. 2014; Zimmerman et al. 2014), which are expected to improve their sensitivity and reliability. To date, however, the presented screening approach represents one of the best strategies for the exposure assessment, and followed the main available technical recommendations on the harmonization of the strategies for measuring exposure to nanoparticles (Methner et al. 2009; Brouwer et al. 2012; Cornelissen et al. 2012; Meier et al. 2013). Further research will focus on the morphological characterization of NPs by SEM-FEG for a complete graphene NPs characterization.

Conclusions

This study was designed to determine the potential graphene particle contamination deriving from the production of GFNs in a specific industrial setting. The obtained information on workplace contamination was then used to estimate the potential workers' exposure to GFNs particles. The research outcomes can be of particular interest because the exposure of workers in real working conditions was assessed with a multi-metric approach (i.e., particle number and mass concentration, particle size distribution, particles diameter, and surface-area concentrations). The multi-metric characterization of occupational exposure to graphene NPs resulted in significantly different results, both for different work environments and for each specific work-task. The study showed that workers are exposed to graphene NPs mean levels lower than the proposed reference values (8-h TWA = 40,000 particle/cm^3) and that the possibility of significant exposure by transient high GFNs particles peaks is not likely. However, the results suggest that workers who are directly involved in some specific work task (material sampling for quality control) have higher

potential for occupational exposure than operators who are in charge of routine production work.

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Conflict of interest

The authors declare that they have no conflict of interest.

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E ALTA TECNOLOGIA
– DiSAT

ASSESSMENT OF AIRBORNE PARTICLES AND AIRBORNE ULTRAFINE PARTICLES CONCENTRATIONS WHILE HANDLING NANOMATERIALS IN CHEMICAL FUME HOOD

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For the attention of
Dr. Laura Giorgia Rizzi

1. INTRODUCTION

Nanomaterials (NMs) are materials containing nano-objects with at least one external dimension in the size range of 1- 100 nm [ISO, 2008]. Due to their peculiar physico-chemical properties, several NMs are produced and used in many industrial sectors [Leso et al., 2017]; among these, the graphene family of nanomaterials (GFNs) has been introduced into several scientific and technological applications [Jang and Zhamu 2008; Rafiee et al. 2009].

To date, for effective precautionary management of potential nanotechnology related risks, a "hierarchy of controls" has been proposed [Engeman et al., 2013]: following this hierarchy (which considers for priority to eliminate/to substitute the use of a hazardous substance and providing workers with adequate information and training), where it is conceivable that occupational exposure may occur, it is necessary to adopt engineering control measures to reduce exposure levels such as the application of local exhaust ventilation, pressure differentials, hoods and enclosed systems [NIOSH, 2016]. In this regard, fume hoods are among the most widespread ventilation systems: airborne particles are captured and removed through the air flow when NMs are manipulated in the fume hood [Tsai et al., 2009]. However, previous evidence has outlined that the handling of NMs in laboratory fume hoods can result in a significant release of airborne particles into the laboratory environment and in the operator's breathing zone [Tsai et al., 2009].

Then, the preliminary hypothesis of this study was that the handling of graphene nanoplatelets in fume hoods may cause exposure arising from the emission and dispersion of graphene particles, mainly in the form of airborne ultrafine particles ("UFP," i.e., particles with diameter < 100 nm), but also in the form of fine particles ("FP", i.e., particles with diameter between 0.1 and 1 μm) or coarse particles ("CP"; i.e., particles with diameter > 1 μm).

The main objective of the study is to assess the effectiveness of typical fume hoods in protecting against graphene NM exposure and to define the conditions that result in lower dispersion of particles in the chemical hood and in the laboratory environment and less exposure of the operators.

2. METHODS

Although different standard methods have been developed to evaluate hood performance and the potential for contaminant leakage [BSI 1994; CEN 2003; ANSI/ASHRAE 1995; ANSI 2003], these methods typically rely on a tracer gas with no worker present, so their applicability to the actual handling of nanoparticles in a fume hood is uncertain [Tsai et al., 2009]. Therefore, an experimental protocol was established based on previous studies concerning similar tests on handling NMs in fume hoods [Tsai et al., 2009, 2010; Dunn et al., 2014].

Nanomaterial and hood

For this study an extremely fine nanopowder of pristine graphene nanoplatelets was used (Directa Plus srl, Lomazzo – Italy; Trade name "Directa Plus - Pure G+"). The pristine graphene nanoplatelets have lateral dimensions of a few micrometers and thickness of a few nanometers; the nanoplatelets are weakly aggregated by van der Waals forces, exhibiting an apparent density of approximately 60 g/L. Particle handling was studied using a constant velocity hood (Typhoon Twin - model 2003HIC; Labosystem srl, Rovellasca - Italy) that uses a controller to vary the fan speed as the sash is moved to maintain a constant hood face velocity.

NM handling and cleaning of the hood

NM handling was performed in each hood by transferring or pouring 100 g of the investigated material from beaker to beaker (3000 ml beakers were used). The transferring task was performed by using a spatula to transfer nanoparticles from one beaker to another beaker, while for the pouring task, nanoparticles were poured directly from one beaker into a second beaker at the center of the open top (the beakers were adjacent to each other at the open edge). After completion of the NM handling activities, cleaning of the work surface inside the hood was performed with laboratory paper moistened with water (wet wiping). A standardized protocol was used to perform the handling and cleaning tasks: all operations were performed sequentially, following a fixed schedule and at predetermined times: each single simulation / monitoring session had a total duration of 35 minutes (Table 1). In addition to the operations described

above, a 5-minute interval ("5-min lag") was provided at the end of each single operation to evaluate the possible re-suspension and/or deposition of PM and UFP due to the performed activities. During these lag periods, the front glass of the hood and the suction front speed were left under the conditions provided for the test. For each test, prior to the beginning of nanomaterial manipulation, PM and UFP background concentrations were measured at each monitoring site (i.e., natural and anthropogenic particles occurring in the workplace air considered in a non-perturbed condition).

Table 1. Time schedule for the operations performed for each test.

Timing (minutes)	Task	Description
0-5	Background concentrations	Determination of PM and UFP concentrations prior to start of activity
5-10	Handling NM (Transferring)	Transferring of 100 g of NM from one beaker to another by spatula
10-15	5-min lag (Transferring)	Lag period - evaluation of the suspension and / or deposition of PM and UFP associated with the previous operation
15-17	Handling NM (Pouring)	Transferring of 100 g of NM from one beaker to another
17-22	5-min lag (Pouring)	Lag period - evaluation of the suspension and / or deposition of PM and UFP associated with the previous operation
22-25	Cleaning	Cleaning of the work surface inside the hood (wiping)
25-30	5-min Lag (Cleaning)	Lag period - evaluation of the suspension and / or deposition of PM and UFP associated with the previous operation
30-35	Final concentrations	Determination of PM and UFP concentrations at the end of the handling and cleaning activities

Experimental conditions

NM handling simulations have been carried out under different operating conditions. Variables studied in this research include handling method, face velocity and sash position; the simulations and the contextual monitoring were carried out in 3 standardized operating conditions (for a total of 15 replied simulations) as reported in Table 2.

Table 2. Summary of the operating conditions defined for each test.

Test	N of replies	Sash position	Face velocity
#1	5	Low (30 cm)	Medium (0,50 ± 0,05 m/s)
#2	5	High (50 cm)	Low (0,40 ± 0,05 m/s)
#3	5	Low (30 cm)	High (0,65 ± 0,05 m/s)

Since literature sources recommend that sash height should be kept as low as possible when manipulating NMs and to operate with a face velocity between 0.4 and 0.6 m/s [Tsai et al., 2009; Dunn et al., 2014]; according to the initial hypothesis, the operating conditions defined for test # 1 (sash height: 30 cm; face velocity: 0.50 ± 0.05 m/s) could be considered optimal conditions for NM manipulation in a chemical hood. Consequently, the operational conditions defined for test # 2 (sash height: 50 cm; face velocity: 0.40 ± 0.05 m/s) should be considered potentially less precautionary than those defined for test # 1. In contrast, the operating conditions defined for test # 3 (sash height: 30 cm; face velocity: 0.65 ± 0.05 m/s) could be considered potentially more precautionary than those defined for test #1 [Tsai et al., 2009; Dunn et al., 2014].

Real-time particle measurement

The measuring strategy consisted of: (i) determination of the "background" particle number concentrations (PNC); (ii) determination of the particle number concentration during the handling and cleaning activities by means of micro-environmental and personal measurements; (iii) distinction of operation-related GFN particles from background aerosols. Average airborne PNC was measured in each location before the handling of nanomaterial to obtain an average background number concentration, which is then subtracted from the measurements made during the subsequent activities;

this approach basically assumes that the concentration determined in each location during no-activity periods is representative of the background concentration, and any increased concentrations during the work activity can be attributed to the NM processing [Kuhlbusch et al., 2011]. Once the background particle number concentrations have been determined, specific measurements are made simultaneously at different locations: GFN particle concentrations were estimated by comparing the measured concentrations with the background concentration, following different approaches for background distinction. However, note that these approaches are considered as a proxy for assessing NM airborne concentrations and exposure levels, despite there being several possible errors associated with use of count-difference methods for background distinction [Kuhlbusch et al., 2011; Berges et al., 2014; Spinazzè et al., 2016]. In this study, a precautionary and conservative approach was adopted, attributing the whole differential particle concentration as GFN particles (without any differentiation between incidental and process-related engineered nanoparticles).

Environmental measurements were taken at a laboratory room location ("laboratory") and at the source location ("hood"); personal measurements were collected in the breathing zone of the operator who conducted the handling simulations ("operator"). Laboratory particle concentrations were measured 2 m in front of the hood, at the center of the room, and at the level of the respiratory tract (150 cm - standing adult person). Particle concentrations at the source were measured at the downstream side of the releasing source (instruments were placed at 15 cm vertically above the beaker at the downstream edge); NM handling tasks were performed in the hood at the center of the work surface. A time-activity diary was also used to separate the continuous data as a function of the different monitored tasks. All the instruments were used simultaneously for the entire length of the monitoring period.

Numerical concentrations of airborne UFP were measured using a miniature diffusion size classifier (DSC) and condensation particle counters (CPCs). The DSC used for this study (DiSCmini, Matter Aerosol AG, Wohlen AG, Swiss) measures the particle number concentrations (range: 10^3-10^6 particle/cm^3) and the particles' average diameter in the size range of approximately 10-700 nm [Fierz et al. 2011]. The DSC also estimates with

reasonable accuracy the lung-deposited surface area concentration (LDSA), defined as the particle surface area concentration per unit volume of air, weighted by the deposition probability in the lung and calculated according to ICRP report 66 [Fierz et al. 2011]. Portable CPCs (P-Trak Ultrafine Particle Counter model 8525; TSI Inc., Shoreview, MN, USA) were also used in this study to quantify the airborne UFP number concentration (size range: 0.02 to 1 μm). Finally, numeric concentrations of PM were also measured using optical particle counters ("OPC," mod. Handheld 3016, Lighthouse Worldwide Solutions, Fremont, CA, USA), which can provide real-time measurement of particles with optical diameter for six different dimensional fractions in the 0.3-10 μm range. For this study results obtained with the OPC were referred to two cumulative dimensional fractions: fine particles (PM <1 μm) and coarse particles (PM > 1 μm). Data were measured with a 1-s (CPC, DSC) or 10-s frequency (OPC). Table 3 summarizes the monitoring design and strategy. It should be noted that the combined characterization of CP (PM > 1μm), FP (PM < 1 μm) and UFP (<100 nm) can provide information on the nature and magnitude of potential contamination of the environment and of the professional exposure to nanomaterials. In fact, this experimental design provides complete aerosol evaluation and allows the presence of sources of nanomaterials and aggregates of nanomaterials (also of micrometric size) to be identified with reasonable certainty [Methner et al., 2009].

Table 3. Monitoring strategy and instruments used for measurements

Monitored task	**PM fraction**	**Location**		
		Laboratory	**Hood**	**Personal**
Background Concentrations (site-specific)	UFP	CPC (PNC)		DSC (PNC, mean diameter, LDSA)
	FP CP	OPC (PNC, size-resolved)		
Handling and cleaning operations	UFP	CPC (PNC)		DSC (PNC, mean diameter, LDSA)
	FP CP	OPC (PNC, size-resolved)		

3. RESULTS

The variations of airborne UFP and PM concentrations from background concentrations are reported in Table 4 and Table 5, and in Figure 1-3. The background number concentrations defined for each location were subtracted from the measurements made during handling of NMs; thus, results from this type of measurement should be interpreted as variation in the concentrations of airborne NM particles.

Table 4. Absolute and relative (%) average variation of UFP concentrations from the background value. The results are calculated from 5 replicates of each test.

Test		PNC - Hood [particle/cm^3]	PNC - Operator [particle/cm^3]	Mean Diameter - Operator [nm]	LDSA - Operator [µm^2/cm^3]	PNC - Laboratory [particle/cm^3]
#1	**Background (mean)**	**6785**	**5497**	**93.8**	**26.87**	**6446**
	Transferring	-190 (-7.2)	52 (1.9)	0.3 (0.4)	0.28 (1.0)	26 (0.3)
	5-min lag (Transferring)	-397 (-8.1)	-231 (-0.1)	0.1 (0.01)	0.13 (0.6)	-387 (-4.2)
	Pouring	-580 (-9.9)	-515 (-3.4)	-0.2 (-0.4)	-0.17 (-0.4)	-440 (-3.3)
	5-min lag (Pouring)	-641 (-13.4)	-651 (-5.1)	-0.1 (-0.4)	-0.11 (-0.1)	-735 (-7.4)
	Cleaning	-776 (-21.7)	-834 (-7.8)	-0.5 (-0.9)	-0.54 (-1.8)	-753 (-6.3)
	5-min lag (Cleaning)	-771 (-21.3)	-710 (-2.8)	-0.6 (-1.2)	-0.63 (-1.9)	-133 (7.7)
	Final concentration	-782 (-10.1)	-509 (3.8)	-0.5 (-1.1)	-0.51 (-1.3)	-314 (4.7)
	Mean variation ± SD (%)	**-556 ±1155 (-11.4 ± 18)**	**-459 ± 1764 (-1.4 ± 23)**	**-0.2 ± 1.4 (-0.5 ± 2.2)**	**-0.21 ± 1.45 (-0.5 ± 4.8)**	**-362 ± 1882 (-0.7± 27)**
#2	**Background (mean)**	**5748**	**4228**	**102.1**	**24.48**	**5227**
	Transferring	-36 (-0.4)	17 (0.9)	-0.5 (-0.5)	-0.46 (-1.7)	-2 (0.1)
	5-min lag (Transferring)	-120 (-1.8)	27 (0.8)	-0.4 (-0.4)	-0.40 (-1.9)	-11 (0.03)
	Pouring	-67 (-0.7)	65 (2.4)	-0.6 (-0.6)	-0.57 (-2.5)	129 (3.1)
	5-min lag (Pouring)	-41 (-0.1)	190 (6.0)	-0.2 (-0.3)	-0.22 (-0.9)	142 (3.3)
	Cleaning	-45 (-0.3)	197 (5.5)	-0.04 (-0.1)	-0.04 (-0.1)	238 (5.0)
	5-min lag (Cleaning)	-87 (-1.2)	327 (8.7)	0.3 (0.3)	0.31 (1.2)	140 (3.3)
	Final concentration	-122 (-1.99	290 (7.7	0.1 (0.2)	0.13 (0.6)	26 (1.2)
	Mean variation ± SD (%)	**- 74 ± 309 (-0.9 ± 5.8)**	**166 ± 389 (4.8 ± 10.4)**	**-0.1 ± 1.0 (-0.2 ± 1.0)**	**-0.15 ± 1.0 (-0.6 ± 4.2)**	**81 ± 310 (2.0 ± 6.1)**
#3	**Background (mean)**	**4818**	**3828**	**95.7**	**20.69**	**4627**
	Transferring	-43 (-1.0)	-17 (-0.8)	-0.2 (-0.2)	-0.22 (-1.2)	-45 (-0.8)
	5-min lag (Transferring)	-97 (-1.8)	-121 (-2.7)	-0.3 (-0.3)	-0.26 (-1.3)	-30 (-0.4)
	Pouring	-127 (-2..1)	-172 (-3.5)	-0.2 (-0.2)	-0.22 (-0.9)	-73 (-1.0)
	5-min lag (Pouring)	-191 (-3.0)	-226 (-4.3)	-0.4 (-0.5)	-0.44 (-1.8)	-124 (-1.9)
	Cleaning	-304 (-5.3)	-335 (-7.2)	-0.7 (-0.7)	-0.66 (-2.8)	-196 (-3.3)
	5-min lag (Cleaning)	-278 (-4.6)	-332 (6.9)	-0.6 (-0.6)	-0.57 (-2.3)	-172 (-2.8)
	Final concentration	-341 (-5.9)	-290 (-5.5)	-0.7 (-0.7)	-0.70 (-2.9)	-89 (-0.8)
	Mean variation ± SD (%)	**-200 ± 443 (-3.4 ± 8.6)**	**-209 ± 549 (-4.3 ± 13.7)**	**-0.4 ± 1.2 (0.5 ± 1.3)**	**- 0.44 ± 1.20 (-1.9 ± 5.9)**	**-101 ± 418 (-1.5 ± 8.9)**

Table 5. Absolute and relative (%) average variation of fine particulate (FP; 0.1-1 μm) and coarse particulate (CP; > 1 μm) concentrations from the background value. The results are calculated from 5 replicates of each test.

Test		FP - Hood	CP- Hood	FP - Operator	CP - Operator	FP - Laboratory	CP - Laboratory
#1	**Background (mean)**	**32.7**	**0.91**	**43.9**	**0.24**	**39.0**	**0.15**
	Transferring	0.8 (4.6)	3.72 (775)	1.9 (3.6)	0.09 (41)	1.4 (2.9)	0.06 (39)
	5-min lag (Transferring)	2.8 (4.6)	0.11 (36)	-1.0 (-3.5)	0.05 (22)	-0.2 (-1.2)	0.04 (26)
	Pouring	3.6 (12.7)	3.61 (525)	1.7 (2.3)	0.12 (57)	0.3 (0.1)	0.05 (39)
	5-min lag (Pouring)	2.7 (14.9)	0.29 (71)	-0.8 (-3.7)	0.07 (34)	-0.5 (-2.1)	0.05 (33)
	Cleaning	1.9 (12.3)	0.32 (72)	-2.5 (-6.8)	0.12 (57)	-1.0 (-3.3)	0.06 (39)
	5-min lag (Cleaning)	0.6 (10.3)	-0.16 (17)	-4.0 (-11)	0.10 (47)	-2.6 (-7.3)	0.05 (35)
	Final concentration	-0.3 (6.2)	-0.34 (-14)	-6.7 (-16)	0.005 (7.1)	-4.0 (-10)	0.04 (26)
	Mean variation ± SD (%)	**1.5 ± 11 (8.5 ± 41.3)**	**0.86 ± 3.03 (188 ± 523)**	**-1.9 ± 5.7 (-5.5 ± 12.3)**	**0.07 ± 1.13 (35 ± 56)**	**-1.0 ± 3.9 (-3.3 ± 9.6)**	**0.05± 0.06 (33 ± 40)**
#2	**Background (mean)**	**38.4**	**0.58**	**47.9**	**0.27**	**43.0**	**0.19**
	Transferring	-1.6 (-4.1)	3.39 (679)	-1.0 (-2.1)	0.01 (5.1)	-0.4 (-1.0)	-0.01(-3.5)
	5-min lag (Transferring)	-1.5 (-3.8)	-0.01 (0.4)	-1.7 (-3.4)	-0.02 (-5.4)	-1.1 (-2.8)	-0.01(-4.0)
	Pouring	-1.9 (-5.0)	1.04 (173)	-1.7 (-3.4)	-0.02 (-5.9)	-1.4 (-3.5)	-0.01 (-2.4)
	5-min lag (Pouring)	-0.6 (-1.5)	0.01 (3.7)	-1.5 (-2.8)	-0.02 (-2.5)	-1.1 (-2.8)	-0.01 (-3.7)
	Cleaning	0.3 (0.8)	0.08 (19)	1.2 (2.3)	0.00 (5.9)	-0.3 (-0.9)	-0.01 (-4.6)
	5-min lag (Cleaning)	-0.4 (0.8)	0.04 (9.1)	-0.4 (-0.8)	-0.01(1.7)	-0.7 (-1.7)	0.01 (4.5)
	Final concentration	-0.5 (-0.8)	-0.02 (-2.7)	-2.3 (-4.4)	0.003 (2.6)	-1.2 (-2.6)	0.01 (4.2)
	Mean variation ± SD (%)	**-0.8 ± 3.7 (-2.1 ± 9.9)**	**0.64 ± 2.19 (128 ± 472)**	**-1.2 ± 3.0 (-2.3 ± 6.1)**	**-0.01 ± 0.09 (-0.2 ± 32)**	**-0.9 ± 2.2 (-2.1 ± 5.3)**	**-0.004± 0.05 (-1.1 ± 24)**
#3	**Background (mean)**	**44.4**	**0.59**	**54.2**	**0.27**	**50.2**	**0.16**
	Transferring	-1.9 (-4.1)	3.92 (838)	-1.0 (-1.2)	-0.04 (-10)	-1.4 (-2.2)	-0.01 (-6.7)
	5-min lag (Transferring)	-1.8 (-3.4)	0.11 (25)	-1.8 (-2.2)	-0.06 (-17)	-2.0 (-2.6)	-0.02 (-11)
	Pouring	-2.6 (-4.6)	1.08 (197)	-2.2 (-2.7)	-0.03 (-7.7)	-2.7 (-3.7)	-0.02 (-7.8)
	5-min lag (Pouring)	-2.5 (-3.9)	-0.04 (-1.5)	-3.3 (-4.2)	-0.06 (-18)	-3.4 (-4.6)	-0.02(-9.0)
	Cleaning	-2.4 (-3.6)	0.02 (11)	-3.2 (-3.6)	-0.04 (-14)	-4.1 (-5.6)	-0.02 (-12)
	5-min lag (Cleaning)	-2.5 (-3.3)	-0.08 (-6.7)	-3.3 (-3.2)	-0.05 (-13)	-4.1 (-5.4)	-0.01 (-6.4)
	Final concentration	-2.9 (-3.4)	-0.17 (-24)	-3.4 (-2.7)	-0.08 (-24)	-4.5 (-5.6)	-0.02 (-11)
	Mean variation ± SD (%)	**-2.3 ± 4.0 (-3.7 ± 8.5)**	**0.69 ± 2.51 (152 ± 582)**	**-2.6 ± 5.8 (-2.8 ± 8.3)**	**-0.05 ± 0.09 (-15.4 ± 28)**	**-3.1 ± 5.5 (-4.2 ± 8.8)**	**-0.02± 0.04 (-9.1 ± 22)**

Figure 1: Absolute variation from the background value (set equal to 0, continuous grey line) of UFP concentrations measured within the chemical hood (dot-dash black line), personal exposure level (dashed black line) and laboratory (continuous black line) in three operating conditions (a = Test #1; b = Test #2; c = Test #3). The data are expressed as the average value recorded for each simulation phase (5 replicates for each operating condition). Vertical bars indicate the 95% confidence interval.

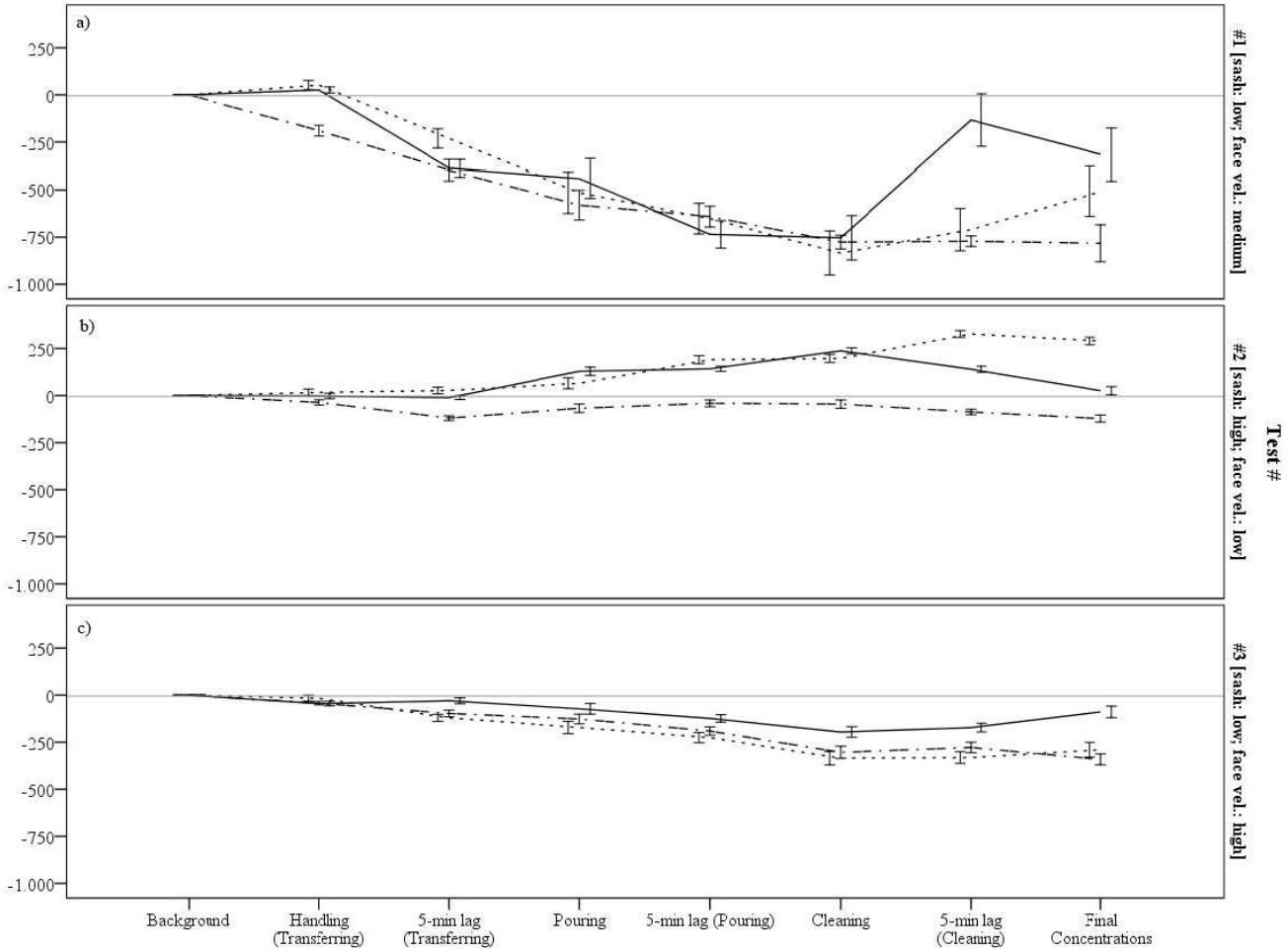


Figure 2: Absolute variation from the background value (set equal to 0, continuous grey line) of FP concentrations measured within the chemical hood (dot-dash black line), personal exposure level (dashed black line) and laboratory (continuous black line) in three operating conditions (a = Test #1; b = Test #2; c = Test #3). The data are expressed as the average value recorded for each simulation phase (5 replicates for each operating condition). Vertical bars indicate the 95% confidence interval.

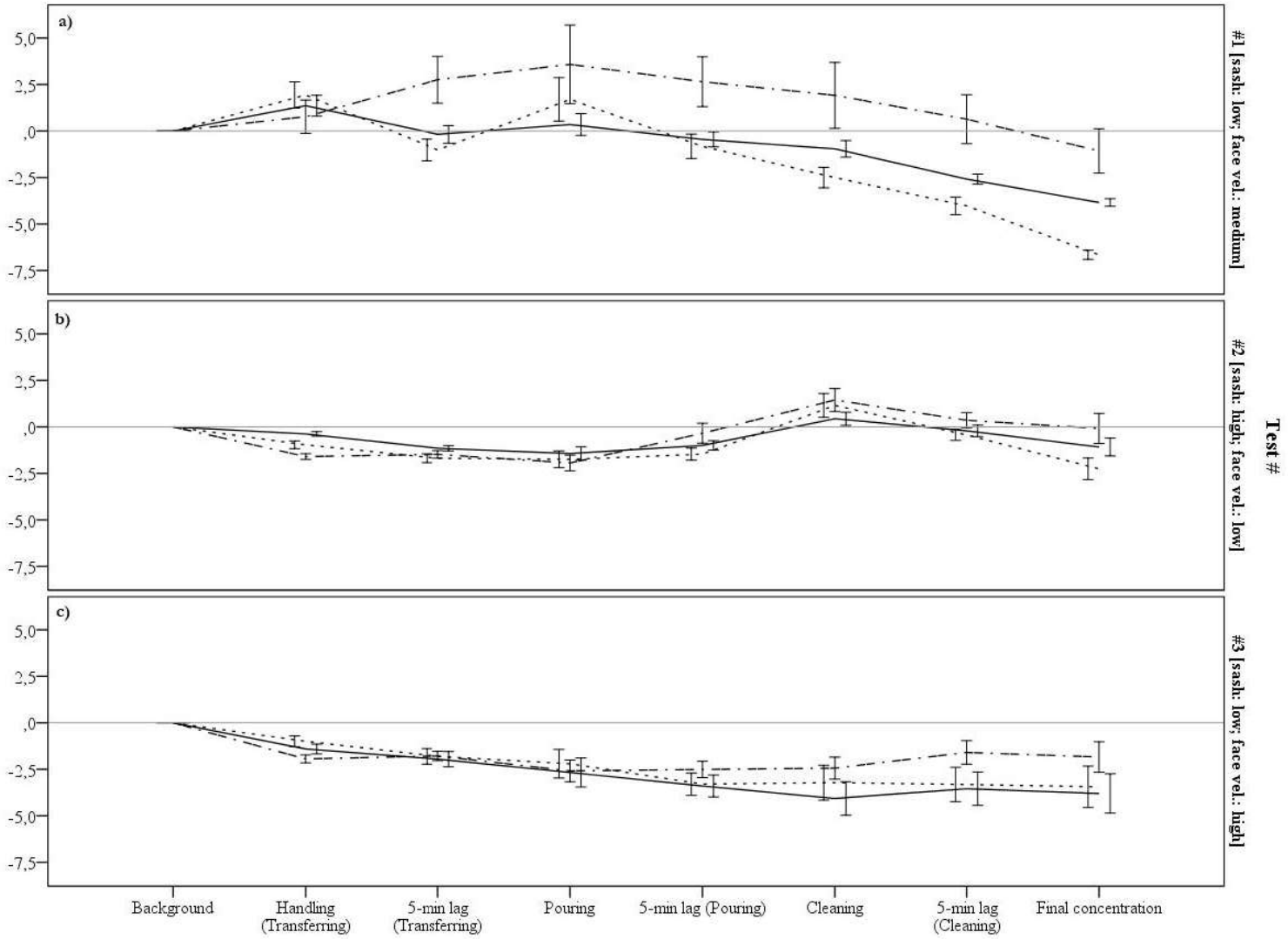
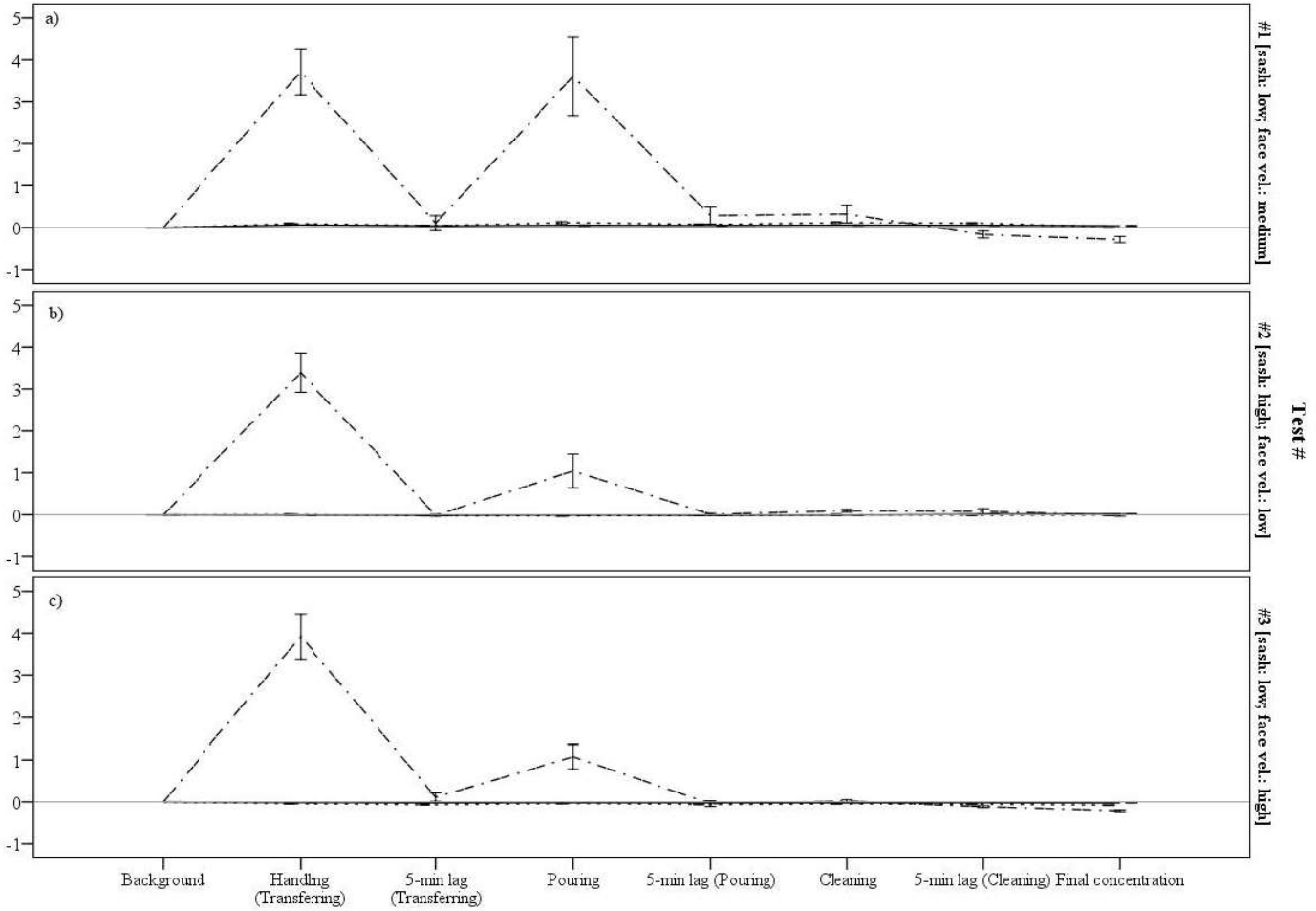


Figure 3: Absolute variation from the background value (set equal to 0, continuous grey line) of CP concentrations measured within the chemical hood (dot-dash black line), personal exposure level (dashed black line) and laboratory (continuous black line) in three operating conditions (a = Test #1; b = Test #2; c = Test #3). The data are expressed as the average value recorded for each simulation phase (5 replicates for each operating condition). Vertical bars indicate the 95% confidence interval



Test #1.

Results for Test #1 outlined a general reduction of mean UFP concentrations (mean ± standard deviation; %) when compared to the background value measured at the three monitored stations: hood (-556 ± 1155 particle/cm^3; -1.4 ± 18%), operator (-459 ± 1764 particle/cm^3, -11.4 ± 23%) and laboratory (-362 ± 1882 particle/cm^3; -0.7 ± 27%). However, no relevant variations of the mean diameter or LDSA were observed in association with the reduction of PNC. By analyzing the variations in UFP concentrations recorded for the various tasks performed during test #1 (Table 4, Figure 1a), it should be noted that only the transferring task involved a slight increase in the concentrations measured for the operator (52 particle/cm^3, 1.9%) and laboratory (26 particle/cm^3, 0.3%); however, this increase is followed by a sharp decrease in concentrations during the next observation period (5 min lag - transferring). Subsequently, after the pouring task, a slight increase in UFP concentrations was observed at the laboratory site and for operator exposure, which, however, was not sufficient to cause a new overrun of the initial background value, as seen in Figure 1a. Only after cleaning the hood's work surface did UFP concentrations decrease again at these two monitoring stations. On the other hand, UFP concentrations showed a regular and stable reduction in the "hood" site, contributing to the hypothesis that the operational conditions for test # 1 are adequate to ensure efficient removal from the hood compartment of UFP, which can eventually re-suspended during the handling of the NM. It should be noted that overall, at the end of the test, UFP concentrations at the three measurement sites are consistently lower than the background concentrations. Concerning FP and CP concentrations, different behavior could be observed (Table 5, Figure 2a e 3a). Although the results outlined overall reductions of FP concentrations in personal exposure (-1.9 ± 5.7 particle/cm^3; -5.5 ± 12.3%) and in the laboratory site (-1.0 ± 3.9 particle/cm^3, -3.3 ± 9.6%), increases in CP concentrations were observed for these same two positions (operator: 0.07 ± 1.13 particle/cm^3; 35 ± 56%; laboratory: 0.05 ± 0.06 particle/cm^3; 33 ± 40%), as was an increase in PM concentrations at the hood monitoring site (FP: 1.6 ± 11 particle/cm^3; 8.5 ± 41.3%; CP: 0.86 ± 3.03% particle/cm^3; 188 ± 523%). This increase can be attributable to transferring and pouring activities, which involve a significant increase in fine particulate concentrations and

especially of coarse particulate matter. However, as is apparent from Figures 2a and 3a, this increase in concentrations is efficiently offset by the "lag" period provided after the NM handling and subsequently by the cleaning of the work surface; at the end of the test these two procedures contributed to the attainment of UFP and PM concentrations that were lower or comparable to the background values. Therefore, although not-negligible increases in UFP and PM concentrations were observed during the NM handling tasks, the operating conditions for Test #1 are adequate to ensure efficient removal of particles possibly raised during the handling of the NM, especially if appropriate compensation times are set after these operations.

Test #2.

In contrast to the above, for Test #2 an increase in mean UFP concentration compared to the background value was observed for the operator exposure (166 ± 389 particle/cm^3, 4.8 ± 10.4%) and at the laboratory site (81 ± 310 particle/cm^3, 2.0 ± 6.1%), while a simultaneous decrease in UFP concentration at the hood position was observed (-74 ± 309 particle/cm^3; -0.9 ± 5.8%) (Table 4, Figure 1b). No relevant variations of the mean diameter or LDSA of the particles were observed. Interestingly, UFP concentrations at the hood position never showed an increase over the background value, thus corroborating the hypothesis that the operating conditions for Test #2 are still adequate to ensure efficient removal of UFP possibly raised during the handling of the NM. However, for the operator exposure and at the laboratory site, average concentration values are constantly higher than the background values, and they showed a moderate but steady increase between the transferring and cleaning tasks. Only after cleaning the hood work surface did the UFP concentrations measured at the laboratory site return to values comparable to background concentrations (i.e., resulting in an average increase of 26 particle/cm^3, which is equivalent to an average increase of 1.2%), while personal exposure resulted in an increase of approximately 300 particle/cm^3 (equivalent to an 8% increase) with respect to background concentrations. Regarding FP and CP concentrations (Table 5, Figures 2b and 3b), an overall reduction of FP concentration was established for personal exposure (-1.2 ± 3.0 particle/cm^3; -2.3 ± 6.1%) and for the laboratory site (-0.9 ± 2.2 particle/cm^3; -2.1 ± 5.3%). For these two same sites, a reduction of CP concentrations (personal: -0.01 ± 0.09 particle/cm^3; -0.2 ±

32%; laboratory: -0.004 ± 0.05 particle/cm^3; -1.1 ± 24%) was also observed. At the hood site a reduction of average FP concentrations (-0.8 ± 3.7 particle/cm^3; -2.1 ± 9.9%) and a simultaneous increase in average CP concentrations (0.64 ± 2.19 particle/cm^3, 128 ± 472%) were observed. The increase of CP could be attributable to NM handling operations (transferring and pouring). However, even in this case (as is clearly shown in Figure 3b), the increase in concentrations is efficiently offset during the lag period expected after the material handling and, subsequently, with the cleaning operations of the hood's work surface. At the end of the test PM concentrations were lower than (or comparable to) the background values. Thus, considering results obtained during NM handling performed under Test #2 operating conditions, the potential for dispersion of NMs in the hood compartment and in the laboratory environment, as well as potential exposure of the operator involved in the manipulation of nanomaterials, cannot be excluded.

Test #3.

Analogously to what was observed for Test #1, measurements for Test #3 generally showed a reduction with respect to background for UFP concentrations at the three monitored stations: hood (-200 ± 443 particle/cm^3; -3.4 ± 8.6%), operator (-209 ± 549 particle/cm^3, -4.3 ± 13.7%) and laboratory (-101 ± 418 particle/cm^3; -1.5 ± 8.9%). In association with this reduction of PNC, a non-negligible variation of the particle LDSA (-0.44 ± 1.20 μm^2/cm^3; -1.9 ± 5.9%) was also observed. By analyzing the variations in UFP concentration observed for the various tasks of Test #3 (Table 4, Figure 1c), none of the operations performed resulted in an increment of UFP concentration in any of the investigated locations; conversely, the reduction of UFP concentrations appears to be characterized by a steady and constant trend; a slight increase in the concentrations at the laboratory and hood site was observed because of the cleaning activity. This last was not enough to determine an exceedance of the initial background value anyway. Overall, at the end of the test, the UFP concentrations at the three measurement sites were significantly lower than the background values. This evidence corroborates the hypothesis that the conditions for Test #3 are adequate to ensure efficient removal of UFP during the handling of NM. A reduction of FP concentrations was also determined for personal exposure (-2.6 ± 5.8 particle/cm^3; -2.8 ± 8.3%) and the laboratory site (-3.1

± 5.5 particle/cm^3; -4.2 ± 8.8%). At the same positions, a reduction of CP concentrations was also observed (operator: -0.05 ± 0.09 particle/cm^3, -15.4 ± 28%, laboratory: -0.02 ± 0.04 particle/cm^3; -9.1 ± 22%). In the hood site, a reduction of average FP concentration (-2.3 ± 4.0 particle/cm^3; -3.7 ± 8.5%) and a simultaneous increase in CP concentration (0.69 ± 2.51 particle/cm^3, 152 ± 582%) were also observed. As discussed above, this increase is mainly attributable to transferring and pouring activities; however, even in this case (as shown in Figure 3c), the increase in concentrations is efficiently offset during the lag period expected after the NM handling and then with the cleaning of the work surface. At the end of the test, FP and CP concentrations were lower than the background values. In summary, although non-negligible increments of PM concentrations have been observed during the NM handling tasks, the above operating conditions could be considered adequate for NM handling in a chemical hood; further, Test #3 conditions appeared to ensure efficient removal of particles eventually re-suspended because of NM handling, especially if adequate compensation times are provided after such activities.

4. DISCUSSION

In the present study, the intensity of UFP and PM contamination (and therefore of the potential exposure) resulting from handling of NM in a fume hood depends on several factors, primarily the face velocity and the sash height of the hood. The performed tests were characterized by distinct values for these two variables with the intention of simulating three scenarios characterized by operational conditions considered optimal (test # 1), potentially worse (test # 2) or better (i.e., more precautionary) (test # 3) for handling the studied NM.

In this regard, the results showed that Test # 2 represents a non-precautionary operating condition for which it is not possible to exclude the potential contamination of work environments by UFP and PM generated by the handling of NM. This statement is based mainly on the observed increase in UFP concentrations in the laboratory and at the level of personal exposure, a behavior in contrast to what was observed during the other simulations. On the other hand, Test # 1 showed in general the containment of potential UFP and PM contamination generated by the handling of the NM, but it also showed non-negligible increases in UFP concentrations (i.e., during the transferring task) and PM (Figure S2a). These increments suggest that it would be preferable to adopt more precautionary conditions such as those defined in Test #3. In this latter simulation, increases of UFP and FP concentrations were not observed at any of the three monitoring stations. Concerning CP, non-negligible concentration increases were observed during transferring and pouring operations at the hood position. This increment is effectively compensated for by the lag periods that were defined in the time schedule after the material handling and cleaning activities: this practice contributed to the achievement of final PM concentrations that were lower than the background values. This pattern occurs likewise for all the performed tests regardless of the adopted operating conditions. In this regard, it is possible to state that overall, all tested operating conditions can be considerate adequate to ensure efficient removal of the coarse fraction of PM from the hood compartment during the handling of the NM if appropriate lag periods are respected after such operations. As mentioned, even the cleaning operations of the hood's work surface contributed to the reduction of airborne concentrations of UFP and PM. In this study, wet wiping was adopted: equivalent

systems or more efficient ones (e.g., high efficiency particulate air filter (HEPA) - filtered vacuum cleaners) are equally valid for this purpose.

However, it should be noted that the concentration levels observed for this study are of particularly modest entities. In relation to this latter point, regarding the numerical concentration of nanoparticles, Nano Reference Values (NRVs) have been developed to provide provisional limit values in situations where no recognized exposure limit values are available, as in the case of GFNs. NRVs represent a warning level: when they are exceeded, exposure control measures should be taken. The NRV for the type of material to which this NM belongs (class 2b: bio-persistent granular nanomaterials and fiber-form in the range of 1 to 100 nm, with a density <6.0 g / cm^3) is fixed at 40000 particle/cm^3 as an 8-hour time averaged value [Cornelissen et al., 2012]. Following the measurements made during the tests, the mean total UFP concentrations measured at the laboratory site were between 4541 and 6135 particle/cm^3 with a maximum recorded value of 13200 particle/cm^3, whereas the concentrations measured by personal monitoring were between 3649 and 5103 particle/cm^3 with a maximum value of 12190 particle/cm^3. It therefore emerges that the total average UFP concentrations (including the background aerosol) measured in these operating conditions are in the order of 1/10 (or in any case less than an order of magnitude) of the proposed NRV value. It is also necessary to consider that the most likely way of exposure to nanomaterials is related to brief exposure to transient peak values. In this light, and in the absence of short-term exposure threshold values, a process involving the use of nanomaterials may be considered to pose risk of exposure if (i) short-term exposures exceed three times the reference value for more than a total of 30 min per 8-h working day or (ii) a single short-term value exceeds the reference value by five times. In the present study, no recorded value exceeds the threshold value of 40000 particle/cm^3, so the occupational exposure associated with the handling of the nanomaterials under the investigated conditions may be considered not relevant.

Final considerations

This work discussed a case study meant to provide data on the performance of laboratory hoods when handling NMs for risk assessment purposes. Some limitations in

the study design and methods could have had an impact on the results, including possible errors associated with the use of the count-difference method to estimate particle number concentrations of GFN particles and direct-reading instrument sensitivity. However, the distinction of GFN particles from background aerosols was performed with a conservative approach, and in conclusion, this approach was likely to result in an overestimation of contamination and exposure, as discussed previously [Spinazzè et al., 2016]. In any case, results of this study indicate that the handling of NMs may pose a potential risk of contamination of the work environment and hence exposure of the involved operators. However, some operating conditions can be adjusted to avoid significant personal exposure conditions and contamination of the work environment by the NMs themselves, thus to ensure safer conditions. For this reason, some guidelines are reported below to be considered with the purpose of defining appropriate procedures for the handling of NMs in a chemical hood; these were derived from indications in scientific literature and international guidelines [Leso et al., 2016; Tsai et al., 2009; Tsai et al., 2010; ACGIH 2007; ANSI 2003] revised and integrated in light of the evidence obtained during this study:

- **The face velocity of the chemical hood should be set between 0.4 and 0.6 m/s**. It should be noted that for this study, **the simulation carried out with a frontal velocity of 0.50 ± 0.05 m/s showed consistent results with this indication**, i.e., **these conditions assured adequate containment of the potential UFP and PM contamination generated by the manipulation of the nanomaterial**. **More cautionary conditions can also be considered**: **lower dispersion of particles was observed for a velocity of 0.65 ± 0.05 m/s.** Lower or higher (> 0.8 m/s) frontal velocities can result in dispersion of the NM.
- **The height of the sash must be kept at the lowest possible level** (in the case: 30 cm) while maintaining a proper frontal velocity, as reported in good technical practices;
- **Constant volume chemical hoods** represent an adequate choice for this type of application.

- Handling of NMs in chemical hoods should be carried out by preferring manipulation techniques that involve **the least possible material dispersion and avoid inducing strong turbulence**.
- **Provide a lag period at the end of NM handling**, before proceeding with other activities, to allow the concentration of airborne particles to be reduced in the hood compartment. In this study, a 5-minute lag was sufficient to compensate for increases in particulate concentrations that may have been re-suspended during NM handling.
- **Cleaning the hood's work surface after any NM handling activity**. In this study, wet wiping proved to be an efficient technique; methods equivalent or more efficient (e.g., cleaning with an absolute filter extractor) are equally valid for the reduction of re-suspended PM and UFP concentrations and for containment of the potential contamination.
- **Given the uncertainty related to the toxic potential of GFNs and NMs in general, it is advisable to ensure the protection of workers engaged in the handling and cleaning processes with adequate personal protective equipment** (e.g., filtering half-facemask with (FFP2- or FFP3-class filter), nitrile gloves and protective glasses).

5. CONCLUSIONS

At first, it should be noted that **the concentration levels observed for this study are of particularly modest entities** (i.e., in the order of 1/10 of the proposed reference value), **so the occupational exposure** associated with the handling of the nanomaterials under the investigated conditions **may be considered not relevant**.

Results of this study indicate that the **handling of graphene nanoplatelets may pose a potential risk** of contamination of the work environment - and hence exposure of the involved operators, **if adequate control measures are not taken**. In fact, if inadequate (Test #2) or not sufficiently cautionary (Test #1) operational conditions are used, non-negligible increases in PM and UFP concentrations during the nanomaterial manipulation phases are observed. However, the evidences obtained from the simulations outlined that **the adoption of appropriate operating procedures** (Test #3) **avoids significant personal exposure conditions and contamination** of the work environment by the NM itself, thereby **ensuring safer conditions**.

In this regard, some **guidelines were reported in this study** to be considered **with the purpose of defining appropriate procedures for the handling of NMs in a chemical hood**. These were derived from indications in scientific literature and international guidelines and could be easily applied in any NM-related laboratory scenario to ensure safer conditions. Among the other indication, a particularly important suggestion is to provide a lag period at the end of NM handling, before proceeding with other activities, to allow the concentration of airborne particles to be reduced in the hood compartment. In this study, **a 5-minute lag was sufficient to compensate for increases in particulate concentrations that may have been re-suspended during NM handling**.

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