Toxicity evaluation of the bio-based materials

Deliverable 6.1

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Acronyms	Description
CO	Cotton
D	Deliverable
DPBS	Dulbecco's Phosphate-Buffered Saline
EN	European Standard
GA	Grant Agreement
HPLC	High Performance Liquid Chromatography
ISO	International Organisation for Standardization
l.q.	Limit of Quantification
MI	Internal method
MPs	Microparticles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MX	Mexameter®
NCs	Nanocapsules
PES	Polyester
PHA	Polyhydroxyalkanoate
PLA	Polylactic Acid
PU	Polyurethane
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SLS	Sodium Lauryl Sulphate
SVHC	Substances of Very High Concern
T	Task
TEWL	Transepidermal water loss
TM	Tewameter®
W2BC	**Waste2BioComp**

1. Introduction

This report presents the outcomes of Task 6.1 - Toxicity of the bio-based materials, whose goal was to show that the bio-based materials developed in **W2BC** do not exhibit toxicity. This is a critical point for newly developed materials, to ensure their safety upon manipulation and use, therefore further contributing to their viable entrance in the market.

This task consisted in a comprehensive evaluation of the different toxicity aspects - human health, including skin reactions, but also environmental - of the components developed in the project: bio-based molecules/polymers, capsules and particles, inks, materials (films, composites, foams, printed substrates and coated textiles).

The task was divided in four sub-tasks, each one contemplating different aspects of toxicity:

- T6.1.1: Toxicity of the new bio-based molecules and materials. This sub-task tested selected materials for the presence of hazard chemical substances, using selected test methods of the OEKO-TEX® standard 100 certification, for which CITEVE has a certified laboratory, and other relevant methods, including those being developed in other projects for the detection and quantification of substances on the SVHC candidate list under REACH;
- T6.1.2: Allergic potential. This sub-task tested the potential of the materials that are more prone to come into prolonged contact with the skin to cause allergic reactions. An *in-vivo* study was conducted on healthy volunteers, using corneometry probes to measure erythema and transepidermal water loss (TEWL);
- T6.1.3: Cytotoxicity of the new bio-based molecules and materials. This sub-task tested the effect of the **W2BC** materials when in contact with cells, namely skin fibroblasts, according to ISO 10993-5 (part 12), to assess their cytotoxic potential;
- T6.1.4: Ecotoxicity assessment of the materials during degradation. In this sub-task the germination success and growth of plants that are relevant in agriculture were tested on the substrate after the biodegradation tests done in T6.2.

This task is part if WP6 - Toxicity and sustainability assessment – where the study of toxicity and sustainability (biodegradability and LCSA) aspects were studied for the **W2BC** materials.

This report shows the accomplishment of *Milestone 7 – Bio-based components without toxicity*, by showing that no components developed in the project present significant negative results for any of the toxicity aspects tested.

2. Work carried out in Task 6.1

This section provides a detailed account of the activities done and results obtained under Task 6.1. Each sub-task was addressed systematically to meet the overall objectives of the task.

2.1. T6.1.1 Toxicity of the new bio-based molecules and materials

A key safety concern for new materials is their potential to release harmful substances during use, assuring the safety to the end-users. As previously mentioned, the tests were selected from the testing methods of the OEKO-TEX® standard 100 certification, for which CITEVE is a certified laboratory, and other relevant methods fort restricted substances under REACH (Annex XVII), including those being developed under other projects for the detection and quantification of substances on the SVHC candidate list under REACH regulation. Many of these tests are based on the leaching of nocive compounds during usual handling conditions of the (bio-based) materials, assuring the safety to the end-users.

CITEVE has identified a list of hazard substances (restricted/limited under REACH Regulation) that have some relevance to the **W2BC** materials, presented in Table 1, and some others that could be tested, but were not considered relevant for the project (Table 2). After consulting each partner, and the Safety Data Sheets of some of the products used, a screening of the substances relevant for testing was performed, therefore avoiding a random testing and carrying out unnecessary tests. Besides this screening, the safety data sheets of the substances used in each material were also accessed, from which the following components were highlighted, as these present some hazards (although the substances are not restricted under REACH):

- Inkjet inks:
  - Polyisocyanates (present in the crosslinker, which is a commercial product that follows REACH regulation) – several diisocyanates are restricted, above a certain concentration, under REACH (entries 56 and 74 from Annex XVII);
  - 3,5-dimetilpirazole (CAS 67-51-6): <0.6 wt.% - harmful if swallowed, is suspected of damaging fertility or the unborn child and may cause damage to organs through prolonged or repeated exposure;
- MPs and some NCs:
  - Sodium dodecyl sulphate (CAS 151-21-3) - Acute toxicity (Oral) Category 4, Skin corrosion/irritation Category 2, Serious eye damage/eye irritation Category 2, Specific target organ toxicity - Single exposure [Category 3] Respiratory tract irritation, Specific target organ toxicity - Repeated exposure [Category 2] Kidney;
  - Oregano oil: Acute toxicity, by ingestion, category 4 (1000), Acute, dermal toxicity. Category 3(300), Danger due to aspiration, category 1, Skin corrosion and irritation, category 2, Skin sensitization, category 1, Eye irritation or damage, category 2A;
- PU foams:
  - Bio-based aliphatic polyisocyanate (PDI-trimer) (from 1,5-pentamethylene diisocyanate) – diisocyanates restricted as mentioned above;
- PHA foams:
  - Calcium oxide (CAS 1305-78-8) - causes serious eye damage, causes skin irritation and may cause respiratory irritation;
  - Azodicarbonamide (CAS 123-77-3) – SVHC.

*Table 1 Screening of hazardous substances identified as having relevance for the materials developed in **W2BC**, and identification of which were (or should be) tested in which material (Y: Yes, N: No)*

Substance	PHAs	MPs	NCs	PHA biocomp.	PHA flexible films	PHA foams	PU foams	PHA fibres	PHA spray coatings	PHA knife coatings	Printed substrates
1-methyl-2-pyrolidone	N	N	N	N	N	N	N	N	N	N	N
Chlorinated benzenes and toluenes	N	N	N	N	N	N	N	N	N	N	N
Chloroform	N	N	N	N	N	N	N	Y(1)	N	N	N
Dimethylfumarate	N	N	N	N	N	N	N	N	N	N	N
Formaldehyde	N	N	N	N	N	N	N	N	N	N	Y
N,N-dimethylacetamide	N	N	N	N	N	N	N	N	N	N	N
N,N-dimethylformamide	N	N	N	N	N	N	N	N	N	N	N
Phthalates	N	N	N	N	N	N	N	N	N	N	N
Azodicarbonamide	N	N	N	N	N	Y	N	N	N	N	N
Isocyanates	N	N	N	N	N	N	Y	N	N	N	Y

(1) There is not enough amount of material for testing, and production has been suspended as it does not have scale-up viability.

*Table 2 Screening of hazardous substances that might be tested, but which were not considered as relevant for the materials developed in **W2BC**, and identification of which were (or should be) tested in which material (Y: Yes, N: No)*

Substance	PHAs	MPs	NCs	PHA biocomp.	PHA flexible films	PHA foams	PU foams	PHA fibres	PHA spray coatings	PHA knife coatings	Printed substrates
Alkylphenols	N	N	N	N	N	N	N	N	N	N	Y
Ethoxylated alkylphenols	N	N	N	N	N	N	N	N	N	N	Y
Benzene	N	N	N	N	N	N	N	N	N	N	N

Substance	PHAs	MPs	NCs	PHA biocomp.	PHA flexible films	PHA foams	PU foams	PHA fibres	PHA spray coatings	PHA knife coatings	Printed substrates
Bisphenols	N	N	N	N	N	N	N	N	N	N	Y
Carcinogenic arylamines[1]	N	N	N	N	N	N	N	N	N	N	N
Chloroparafins short chain	N	N	N	N	N	N	N	N	N	N	N
Glycols[2]	N	N	N	N	N	N	N	N	N	N	N
Metals / metal compounds[3]	N	N	N	N	N	N	N	N	N	N	N
PAH[4] / anthracene compounds[5]	N	N	N	N	N	N	N	N	N	N	N
PFOS and its salts, PFOA e its salts, and related	N	N	N	N	N	N	N	N	N	N	N
Preservatives[6]	N	N	N	N	N	N	N	N	N	N	N
Quinoline	N	N	N	N	N	N	N	N	N	N	N

[1] 2-Naphthylammoniumacetate, 4-methoxy-m-phenylene diammonium sulphate, 2,4-diaminoanisole sulphate, 2,4-diaminoanisole sulphate, 2,4,5-trimethylaniline hydrochloride
[2] 2-(2-methoxyethoxy)ethanol (DEGME), 2-(2-butoxyethoxy)ethanol (DEGBE)
[3] Organotin, mercury, cadmium, CR VI, lead, arsenic, nickel, …
[4] Polycyclic aromatic hydrocarbon
[5] Benz[a]anthracene, Benz[e]acephenanthrylene, benzo[a]pyrene, benzo[def]chrysene, Benzo[e]pyrene, Benzo[j]fluoranthene, Benzo[k]fluoranthene, Chrysene, Dibenz[a,h]anthracene]
[6] Phenol (Chlorinated and 2-phenylphenol)

Azodicarbonamide was tested in CITEVE laboratories (result below). CITEVE does not perform the tests for isocyanates, so a survey of laboratories that could perform those was carried out. Only one of the contacted labs performed that test on solid samples, but the quantity of sample requested (10 g of each sample for each isocyanate) was not feasible.

Some hazard substances are also commonly found in components of the inkjet inks, so they were also tested for the different printed samples with indigo ink, to see if the textile substrate or the pre-treatment affected the leaching of these.

Table 3 Samples tested as to the presence of hazard chemicals molecules, test method, and results

Sample	Substance tested	Method	Result
PHA foam 20 ShA	Azodicarbonamide	Oeko-tex 201 M.35:2024v1.2	<100 mg/kg (l.q.)
PHA foam 30 ShA			
PHA foam 40 ShA			
Printed CO fabric with 1% indigo ink, pre-treated with Binder A	• Bisphenols ○ Bisphenol A ○ Bisphenol AF ○ Bisphenol S ○ Bisphenol F ○ Bisphenol B • Formaldehyde • Alkylphenols ○ 4-n-Octylphenol ○ 4-tert-Octylphenol ○ 4-n-Nonylphenol ○ 4-Nonylphenol ○ Sum of Octylphenols ○ Sum of Nonylphenols ○ Sum of Alkylphenols • Ethoxylated Alkylphenols ○ Octylphenoletoxylates ○ Nonylphenoletoxylates ○ Sum of Ethoxyted Alkylphenols	• MI033:rev00 • EN ISO 14184-1:2011 • ISO 21084:2019 • EN ISO 18254-1:2016	○ <6 mg/kg (l.q.) ○ <6 mg/kg (l.q.) ○ <6 mg/kg (l.q.) ○ <6 mg/kg (l.q.) ○ <6 mg/kg (l.q.) • <6 mg/kg (l.q.) ○ <5.0 mg/kg (l.q.) ○ <5.0 mg/kg (l.q.) ○ <5.0 mg/kg (l.q.) ○ <5.0 mg/kg (l.q.) ○ <5.0 mg/kg (l.q.) ○ <5.0 mg/kg (l.q.) ○ <5.0 mg/kg (l.q.) ○ <5.0 mg/kg (l.q.) ○ <5.0 mg/kg (l.q.) ○ <5.0 mg/kg (l.q.)
Printed PES fabric with 1% indigo ink, pre-treated with Binder A			
Printed CO fabric with 1% indigo ink, pre-treated with Biopolymer			
Printed CO fabric with 1% indigo ink, pre-treated with Biopolymer			

As can be seen in the test results (Table 3) none of the tested samples showed the substances above the quantification limits, so we can consider that these materials do not have these substances.

2.2. T6.1.2 Allergic potential

As several substances considered skin sensitizers are not in the Annexes of REACH (Annex XIV or Annex XVII) nor in the SVHC candidate list, the allergic potential of the developed components, in particular of the final materials, as these are the ones that have higher potential to be in direct contact with the skin (producers, sellers, final consumer) have been assessed, by conducting an *in-vivo* study using a group of healthy volunteers, and using corneometry probes to measure erythema and transepidermal water loss (TEWL).

Standard procedures were established, verified by the coordinator and reviewed by the **W2BC** Ethics Advisor, before the work ensued. Documentation including a full description of the work and tests to be carried out were prepared, including procedures for recruitment of volunteers, an informed

consent, the materials to be tested, risks expected and their level, description of the testing methodology and data analysis, size and characteristics of the test population, and non-discriminatory practices.

Materials to be tested

The following materials were selected for testing, as these are the ones that can have continued contacted with the skin, including the controls to be used in the test:

- positive control: sodium lauryl sulphate (SLS) at 10% (w/v). Application of approximately 50 µL on filter paper inside the occlusion chamber
- neutral control: empty occlusion chamber
- testing samples: all in the same day, previously cut into 1 to 1.5 cm diameter discs to fit inside the occlusion chambers:
    - PHA shoe insole
    - PHA spray coated textile
    - Cotton textile printed with indigo ink, biopolymer pre-treatment
    - Cotton textile printed with yellow ink, biopolymer pre-treatment

Risks expected and their level

The study did not involve any health risks; however, it might cause slight skin irritation after the test, which will subside within 24 hours without the need for any treatment. The presence of the following substances in the samples might cause the above-mentioned skin irritation, but the risk is very low:

- PHA shoe insole:
    - Has zinc carbonate and calcium oxide, which may cause skin irritation (mainly when in powder forms), but in very low concentrations;
- PHA spray coated textile:
    - The dispersing agent used in the spray formulation (3 wt.%) has 2-methyl-2H-isothiazol-3-one (0.0015 – 0.0025 wt.%), classified as skin sensitizer cat.1 (H317), but its concentration in the coated textile is negligible;
- Cotton textile printed with indigo ink, biopolymer pre-treatment:
    - The crosslinker used in the ink (3.8%) contains an aliphatic polyisocyanate bloqued (~35%) which may cause skin irritation or when prolonged exposure skin sensitization, the reaction mass of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-2H -isothiazol-3-one (<0,0015 wt.%) which is skin sensitising. However, the final concentration of this component in the printed textile is practically negligible;
    - The wetting and anti-foam agent used (1%) is based on alcohol alkoxylates, which may be mild eye and skin irritant;
    - The indigo pigment is classified as skin irritation cat.2 (H315), but only when in powder form;
    - The dispersing agent used in indigo pre-dispersion in the ink has 2-methyl-2H-isothiazol-3-one (0.0015 – 0.0025 wt.%), classified as skin sensitizer cat.1 (H317), but its concentration in the printed textile is negligible;
- Cotton textile printed with yellow ink, biopolymer pre-treatment:
    - The crosslinker used in the ink (3.8%) contains an aliphatic polyisocyanate bloqued (~35%) which may cause skin irritation or when prolonged exposure skin sensitization, the reaction mass of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-2H -isothiazol-3-one (<0,0015 wt.%) which is skin sensitising. However, the final concentration of this component in the printed textile is practically negligible;
    - The wetting and anti-foam agent used (1%) is based on alcohol alkoxylates, which may be mild eye and skin irritant.

Testing methodology

The influence and interaction with the skin of several **W2BC** samples were evaluated through tests using probes to measure the physical properties of the skin (corneometry). These probes allow the

determination of the effect of the bio-based materials on the skin and validate the impact of the developed materials on sensory comfort, while maintaining the normal condition of the skin surface. The probes used for this assay, considering the purpose of the functionality being developed, are the Tewameter® (TM 300, Courage-Khasaka) e Mexameter® (MX 18, Courage-Khasaka). The Tewameter probe measures transepidermal water loss (TEWL), while the Mexameter probe allows for the measurement of erythema values.

In vivo measurements of TEWL rates can be used to non-invasively monitor changes in the barrier function of the stratum corneum (the outermost layer of the skin). In normal, healthy skin, the barrier is effective, and TEWL rates are typically low. If the barrier is not fully functional, due to toxic or pathological processes, or deterioration from physical or chemical agents, there will be an increase in TEWL values directly related to these alterations. This parameter is indirectly measured by the evaporation density gradient of water from the skin through T and %H sensors. The values are obtained in g/h/m².

Erythema evaluation can also be used as an indicator of skin irritation potential. An increase in erythema levels represents an increase in visible irritation on the skin, characterized by redness. In this case, the measurement is based on light reflection/absorption, and there are no standard/normal erythema values. Therefore, it should always be a comparative analysis (before/after contact with the technical textile). SLS is frequently used as a positive control due to its recognized irritant potential.[1]

Number of volunteers: 10

Test anatomical zone:

- Anterior side of the forearm

Equipment:

- Tewameter® (TM 300, Courage-Khasaka)
- Mexameter® (MX 18, Courage-Khasaka)

Material:

- Positive Control – 10 %(w/v) SLS solution; application of approximately 50 µL onto filter paper inside the occlusion chamber;
- Negative Control – Empty occlusive chamber;
- Samples to be tested;
- Occlusive chamber if 18 mm, Finn Chambers® on Scanpor;
- Adhesive Micropore.

Procedure:

The tests were conducted under stable and controlled environmental conditions: Temperature (20 ± 2) °C and Relative Humidity (65 ± 4) %, and the analysis was performed according to an internal procedure "Evaluation of skin irritant potential," adapted from procedures collected from the literature.[2,3]

1. Initially, three test sites were marked on the inner side of the left forearm and three test sites on the inner side of the right forearm of each volunteer;
2. Then, baseline TEWL and erythema levels were measured at each mark for all volunteers. Two of the marked sites served as controls, and the remaining ones were used for the test samples;
3. Next, the samples and controls will be randomly applied to the volunteers' arms, using occlusive chambers secured to the skin with adhesive for a period of 4 hours;

[1] Tupker, R. A., Willis, C., Berardksca, E., Lee, C. H., Fartasch, M., Atinrat, T., & Serup, J. (1997). Guidelines on sodium lauryl sulfate (SLS) exposure tests. Contact Dermatitis, 37(2), 53–69. http://doi.org/10.1111/j.1600-0536.1997.tb00041.x

[2] Fullerton, A., Fischer, T., Lahti, A., Wilhelm, K.-P., Takiwaki, H., & Serup, J. (1996). Guidelines for measurement skin colour and erythema A report from the Standardization Group of the European Society of Contact Dermatitis. Contact Dermatitis, 35(1), 1–10.

[3] Pinnagoda, J., Tupkek, R. A., Agner, T., & Serup, J. (1990). Guidelines for transepidermal water loss (TEWL) measurement. Contact Dermatitis, 22(3), 164–178.

4. The volunteers will then remove the occlusive chambers and samples from the skin, leaving the area exposed to air for 2 hours;
5. Finally, both erythema and TEWL will be measured again for each volunteer, after contact with the samples;
6. The data was then exported and analyzed.

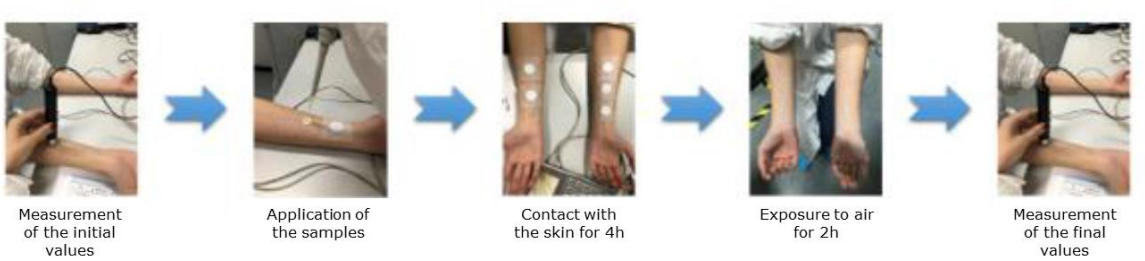

Figure 1 Scheme of the procedure of the corneometry test.

Data analysis

Regarding the treatment of results, the average variations in the difference between final values and baseline values for each sample were calculated for each parameter and across all volunteers. Statistical analysis was also performed to assess the significance of the differences obtained. This analysis involved conducting an ANOVA, which determines the statistical significance of the differences in means across different data sets. One-way ANOVA ($\alpha = 0.05$) was used, considering that the different tested materials represent the only source of variation between results. This allowed us to determine if the source of variation affects the observed results and their amplitude. To conclude the statistical significance of differences between two samples, a Student's t-test was used for the samples and the positive control, which represents a potential irritant. This analysis allowed the assessment of differences between two mean results with unequal variances.

Results

Figure 2 presents the results obtained for the determination of TEWL. The results are expressed as the mean variation in TEWL (g/h/m²) during the contact period for the 10 volunteers involved in the study ($TEWL_{final} - TEWL_{baseline}$).

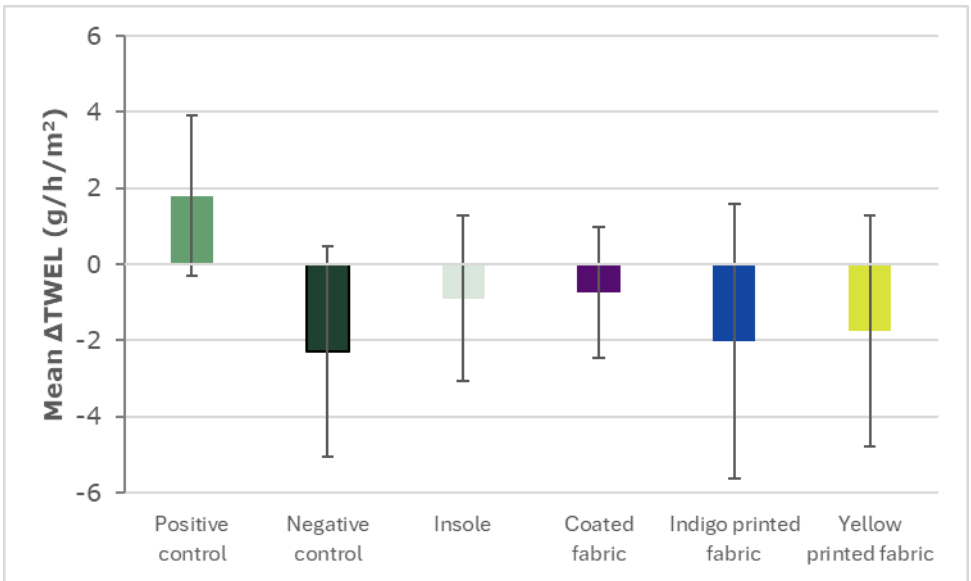

Figure 2 Graphical representation of the mean variation in TEWL as a function of the tested materials. K^+ and K^- represent the positive control (10% SLS) and the negative control (empty occlusion chamber), respectively. Differences observed were significant ($p < 0.05$).

Analysing Figure 2, it can be observed that positive variations indicate an increase in TEWL, which in turn represents a deterioration of the skin barrier function, whereas negative variations indicate a decrease in TEWL, suggesting that the skin barrier function was not affected by the tested materials. The ANOVA analysis detected statistical significance associated with the tested materials as a source of variation for the TEWL parameter, with a confidence level of 95%. It is noteworthy that all volunteers involved in the study had baseline TEWL values ranging between 4.5 and 17.8 g/h/m², indicating a healthy skin condition. Therefore, the conclusions of this study are valid for this state of skin health.

Figure 3 presents the results related to the determination of erythema under the same analysis conditions.

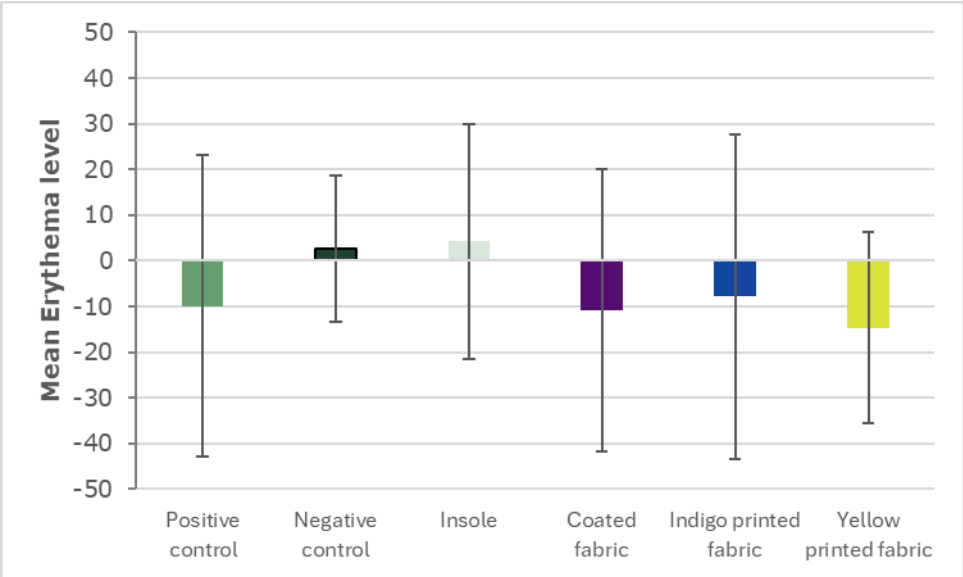

Figure 3 Graphical representation of the mean variation in the erythema level as a function of the tested materials. K^+ and K^- represent the positive control (10% SLS) and the negative control (empty occlusion chamber), respectively.

It was possible to conclude that the samples did not cause significant changes in erythema levels, and therefore none of the tested materials represented a visible irritant potential. Regarding the results obtained for the positive control (SLS), contrary to expectations, the volunteers did not show redness of the skin but instead exhibited flaking with a whitish appearance (Figure 4). Although SLS is widely described in the literature as a cutaneous irritant, the results obtained with the Mexameter probe did not show a significant increase in erythema. This absence may be explained by the type of response induced by SLS, as under certain conditions it may predominantly cause skin flaking and cracking rather than evident erythema. In fact, studies show that even at high concentrations (e.g., 14% SLS under occlusion), the visible inflammatory response may be very mild or even absent in some individuals, being accompanied only by a moderate increase in TEWL. Therefore, in situations where the visible inflammatory response is minimal, TEWL assessment constitutes a more sensitive and reliable marker of skin barrier integrity. In the present trial, the TEWL values confirmed the presence of irritation, supporting the irritating action of SLS despite the absence of significant chromatic changes. Additionally, the response induced by SLS can also be influenced by daily temperature, with lower temperatures being associated with reduced barrier function and hydration, which may once again lead to more aggressive skin reactions, such as epidermal flaking and cracking.

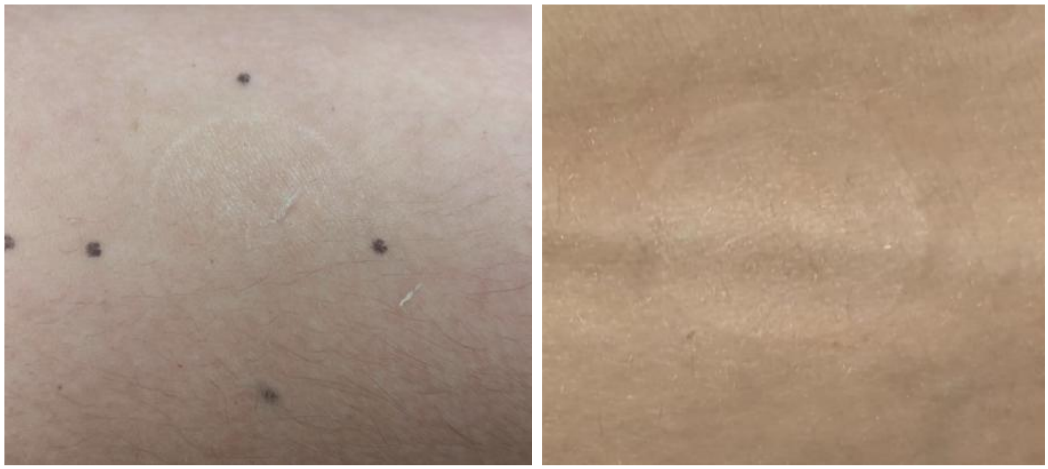

Figure 4 Illustrative images of the mark left by the positive control (10% SLS) after contact with the volunteers' skin.

By analysing the results presented in the graphs in Figure 2 and Figure 3, it was possible to observe significant inter-individual variability in skin behaviour following application of the samples and controls mentioned, which resulted in average parameter values with pronounced standard deviations.

2.3. T6.1.3 Cytotoxicity of the new bio-based molecules and materials

CITEVE has decided to start the cytotoxicity evaluation by conducting simple tests, namely MTT (2,5-diphenyl-2H-tetrazolium bromide) assays according to standard ISO 10993-5, as these are quite simple to accomplish, require very small amounts of the components to test, and is a standard already implement in the certified laboratories at CITEVE.

The MTT method is one of the most used colorimetric assays to evaluate cytotoxicity, based on the activity of mitochondrial enzymes in reducing MTT (yellow in colour) forming purple formazan crystals. The mitochondrial activity of viable cells is constant, so the increase or decrease in the number of viable cells is directly related to mitochondrial activity and, therefore, to the concentration of formazan crystals that are formed. These crystals are subsequently solubilized in isopropanol and can be quantified by reading the absorbance at a specific wavelength, 570 nm.

The tests started with the first indigo dispersion provided by partner PILI. This dispersion was diluted to 5% (the concentration used in the inks – see Deliverable D1.4), and used directly on the cells (100%), or in consecutive dilutions with cell culture media (75%, 50%, 25% and 10%), as defined in the standard. The test was performed on cells L929 and HaCaT. After 24 h of incubation at 37 °C and 5% CO_2, the analysis was performed (as described in the standard). However, due to the intense colour of the indigo pigment, the optical density values obtained were not real (cell viability of 602124% with L929 cells, and 495249% with HaCaT cells), as the value reads were not from the formazan crystal, but mostly from the indigo. Images of this assay can be seen in Figure 5.

The intense coloration of the indigo pigment led to unreliable optical density readings, compromising the accuracy of the MTT assay. Therefore, an alternative method was developed.

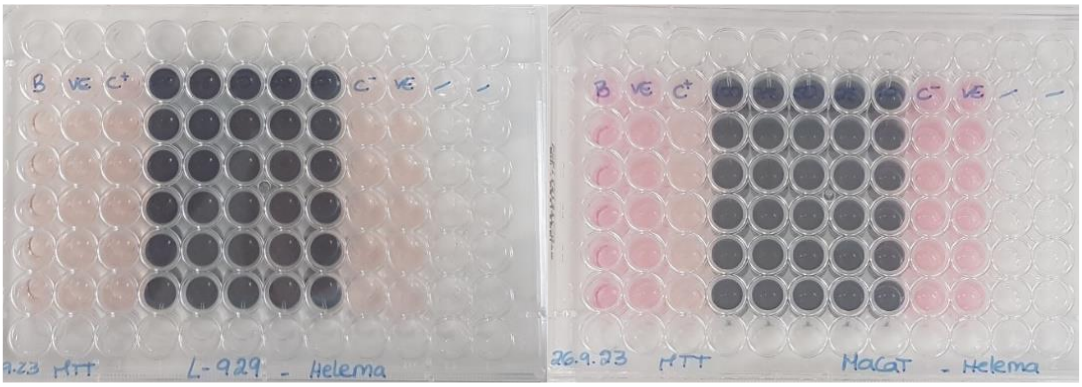

Figure 5 96-well plates used in the MTT assay with the 5% indigo dispersion and cells L929 (left) or HaCaT (right). The intense colour of the indigo is noticeable. Even after the washing steps (not shown), this colour remained, interfering with the optical density readings.

In parallel, a clonogenic assay was performed. This assay measures the ability of a cell to divide and form a colony, defined as containing at least 50 cells. This is a less expensive assay and is therefore widely used as a preliminary method to test the cytotoxic capacity of an agent and the ability of cells to divide and proliferate. Like the MTT assay, the indigo dispersion, diluted to 5% was used directly on the cells (100%), or in consecutive dilutions with cell culture media (75%, 50%, 25% and 10%). The test was also performed on cells L929 and HaCaT. A 7-day incubation followed at 37 °C and 5% CO_2, to observe the effect of the treatments on growth/colony formation. An additional well washing step was carried out with DPBS, and then the cells were fixed with 70% ethanol. Finally, the formed colonies (viable cells) were stained with methylene blue, washed, and left to dry to be visualized.

Although both L929 and HaCaT were able to form colonies for the control condition (Culture Medium), where the cells were in contact with the indigo dispersion, it was difficult to draw conclusions since the pigment marked the wells of the plates and it was not clear to see the cells (Figure 6). Another difficulty observed was the fact that the dispersion was not homogeneous, as can be seen in the images where we have wells with the same dilution, but with very different aspects. Despite all the difficulties, in the wells where there was less staining, related to the pigment, it was possible to see an absence of cells, which could indicate that these treatments being studied exhibit cytotoxicity. However, due to the intense colour of the indigo pigment, it was not possible to count the colonies.

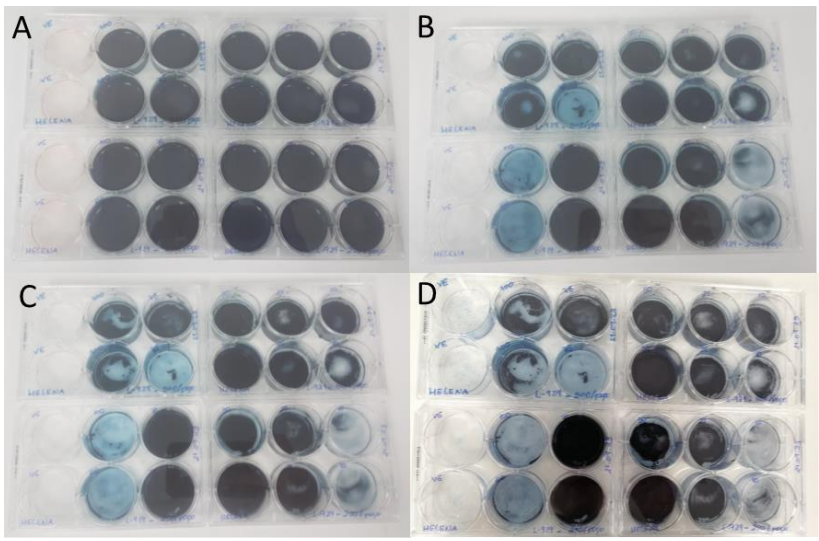

Figure 6 Clonogenic assay with 5% indigo dispersion and L929 cells. A) cells plated and in contact with the 5% indigo dispersion at the different dilutions tested. B) cells after discarding the indigo dispersion at the different dilutions tested. Extreme coloration of the wells may be observed. C) cells after washing the wells 3 times with DPBS. The colouring remains. D) cells after staining with methylene blue. Similar results were obtained with the HaCaT cells.

Given the problems with solubility (and colour in the case of the pigments) of the precursors to be tested (pigments, PHAs and their microcapsules), and to facilitate the comparison with results on the materials to be developed, it was decided that the following tests would be done on extracts of the precursors, instead of using them directly on the cells, following a protocol similar to the one in standard ISO 10993-5 (part 12).

Therefore, in the following tests presented, the precursors were put in an extraction mean (cell culture medium, or artificial acidic sweat, to better simulate the contact of these components with the skin), and then the extracts were incubated with L929 cells (the ones most used) and then a MTT assay was performed. The standard protocol of the extraction is to use 1 g of textile per 10 mL of extraction solution, but as this 1) would require substantial amounts of the components, and 2) when testing the textile or other substrates these components would be in lower concentrations, after a careful analysis of the literature, it was decided to use 10 mg of precursor per mL of extraction medium.

Different samples were tested under ISO 10993-5 (part 12), extracted (10 mg/mL) with perspiration solution (pH 5.5) and cell culture medium (24 h at 37 ºC) and incubated with fibroblasts L929 (24 h at 37 ºC, and 5% CO_2) at 100%, 75%, 50%, and 25% of the extracts if extraction done in cell culture medium or at 33.30%, 22.20%, 14.80% and 9.90% if extraction done in perspiration solution (pH neutralization and addition of cell culture medium prior to adding to the cells, as these do not survive in a media below ~70% of cell culture medium). The samples tested and main results are presented in Table 4. Figure 7 and Figure 8 shows the cell viability for each precursor sample.

As for the PHAs, unprocessed or processed, these did not present any cytotoxic potential, with cell viabilities always above 86% and no cell lysis observed, even for the higher concentrations of the extracts (only PHA.C.3.3.1.1 presented a slightly lower cell viability - 77% - for the extract from the perspiration solution at the higher concentration, but it is still above the **ISO 10993-5 criteria of at least 70% cell viability**).

Similarly, the PHB microcapsules tested have also presented no cytotoxic potential, with cell viabilities above 88% for all concentrations.

However, the pigments did show some cytotoxic potential. The commercial synthetic indigo pigment showed cell viabilities between 55 and 69%, while the indigo from PILI had higher viabilities (above 69%). Despite this, both are considered to have some cytotoxic potential. In the nest phase, printed samples with this pigment were analysed, to see if the cytotoxicity remained (see below).

As for the red pigment (quinacridone), surprisingly, the pigment from PILI had low cell viability (between 1 and 6% for the highest concentrations), while the commercial synthetic pigment had good cell viability, without showing cytotoxic potential. This result indicates that the pigment produced by PILI probably has some contaminant/by-product that is responsible for this high toxicity. As this pigment was later excluded from the project due to scale-up challenges, its cytotoxicity does not affect the project's outcomes.

Table 4 "Precursors" samples tested for their cytotoxic potential under ISO 10993-5, and main results

Sample	Extraction media	Result with cells	HPLC of extract(1)
PHA.A.1.3.1.1	Culture medium* Perspiration solution pH 5.5	Discrete intracytopasmatic granules, no cell lysis, **no reduction of cell growth** ✓ No cytotoxic potential!	Peaks at 22.97, 25.97, 27.58, 30.01 min with λ_{max} 210 nm
PHA.A.2.3.1.1			Peaks at 23.00, 26.00, 26.19, 27.60, 28.17, 30.03, 31.80 min with λ_{max} 210 nm
PHA.A.3.3.1.1			Peaks at 22.99 e 30.04 min with λ_{max} 210 nm
PHA.C.3.3.1.1			Peaks at 22.77, 25.81, 27.41, 29.87 min with λ_{max} 210 nm

Sample	Extraction media	Result with cells	HPLC of extract(1)
MP37 (microparticles of PHB.50.50.Biomer)	Perspiration solution pH 5.5		Peaks at 22.50, 28.47, 31.44 min with λ_{max} 210 nm
PHA.A.2.3.1.1 (processed by UDC)		Not more than 20% of the cells are round, loosely attached and without intracytoplasmatic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable ✓ No cytotoxic potential!	No characteristic peaks detected at λ_{max} 210 nm
Indigo_synthetic		Not more than 70% of the cell layers contain rounded cells or lysed; cell layers not completely destroyed, but more than 50% growth inhibition observable ✓ Cytotoxic potential!	Peak at 37.67 min with λ_{max} 602 nm (direct injection of pigment in acetonitrile gave peaks at 37.74 min)
Indigo_PILI_Nov2023		Not more than 70% of the cell layers contain rounded cells or lysed; cell layers not completely destroyed, but more than 50% growth inhibition observable ✓ Cytotoxic potential!	Peak at 7.23 min with λ_{max} 412 nm (direct injection of pigment in acetonitrile gave peaks at 37.74 min)
Quinacridone_synthetic		Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth ✓ No cytotoxic potential!	Peak at 32.52 min with λ_{max} 289 nm (direct injection of pigment in acetonitrile gave peaks at 32.60 min)
Quinacridone_PILI_ Nov2023		Nearly complete or complete destruction of the cell layers ✓ Cytotoxic potential!	Peak at 32.51 min with λ_{max} 289 nm (direct injection of pigment in acetonitrile gave peaks at 32.50 min)

(1) Performed under gradient conditions on the apparatus: Shimadzu LC-2050C 3D (Kyoto, Japan), with a column: Mediterranea Sea18 5 µm 25×0.46 cm, by using a photo diode array detector between 190 and 800 nm; the gradient conditions were: mobile phase – acetonitrile/water in the ratios 10/90 till 7 min, 100/0 from 35 to 40.5 min, and 10/90 from 40.51 till 50 min, flow rate - 0.4 mL/min, temperature: 30 °C. Extracts were inject without dilution.

Figure 7 Evaluation of cell viability of fibroblasts L929 by the MTT test after 24 h incubation with extracts of different PHAs (10 mg/mL) in acidic solution or cell culture medium, at different concentrations. Differences observed were significant ($p < 0.05$).

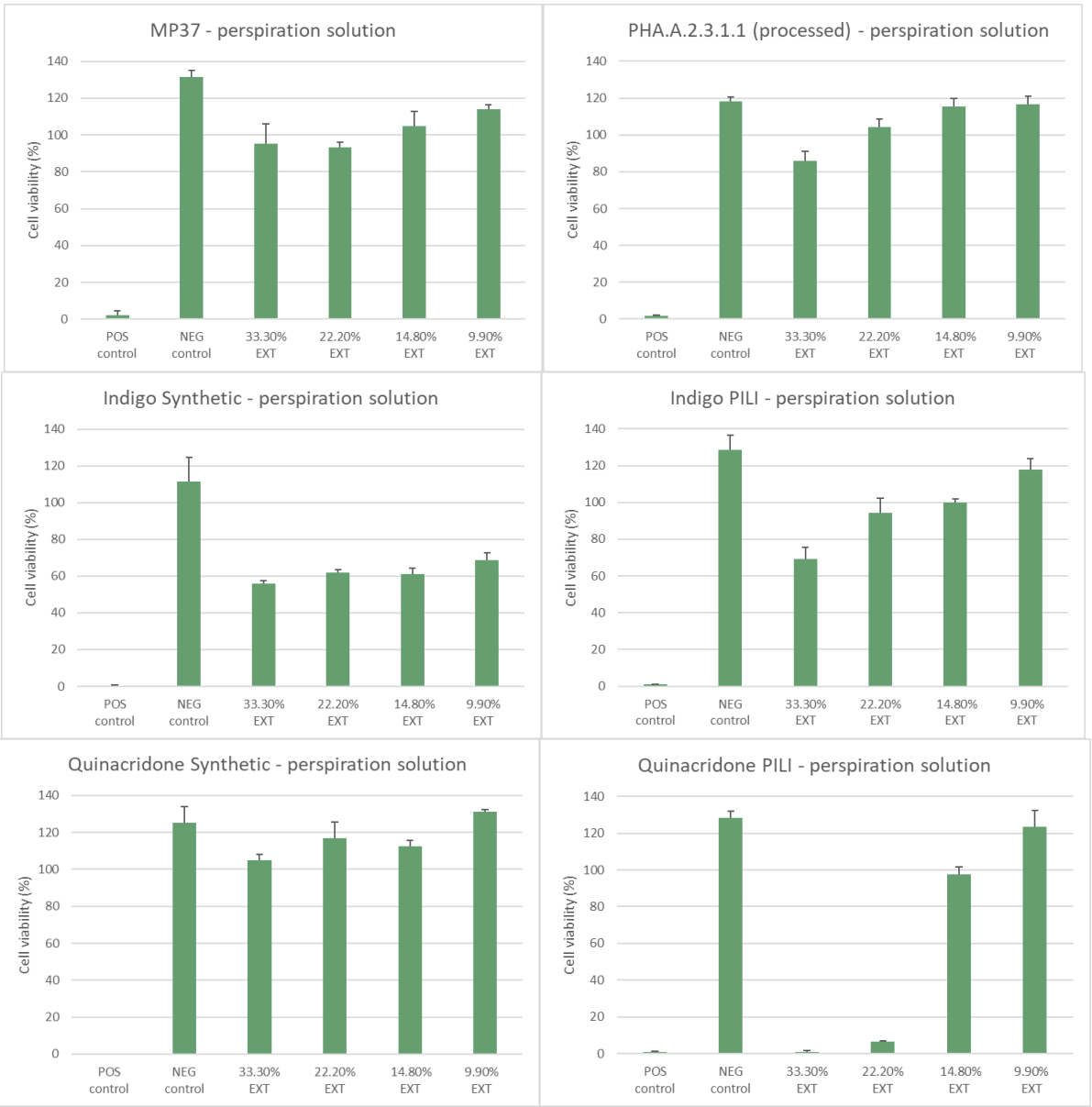

Figure 8 Evaluation of cell viability of fibroblasts L929 by the MTT test after 24 h incubation with extracts of processed PHA, PHB microparticles, indigo pigment (from PILI and synthetic commercial) and red pigment (from PILI and synthetic commercial) (10 mg/mL) in acidic solution, at different concentrations. Differences observed were significant ($p < 0.05$).

The extracts were also analysed by HPLC, to verify if the extraction media contained any substance. The HPLC chromatograms showed that for all tests, some compound(s) was(were) extracted in the media used. In the case of the pigments, it was possible to detect their presence in the extracts. Figure 9 shows some of the HPLC chromatograms.

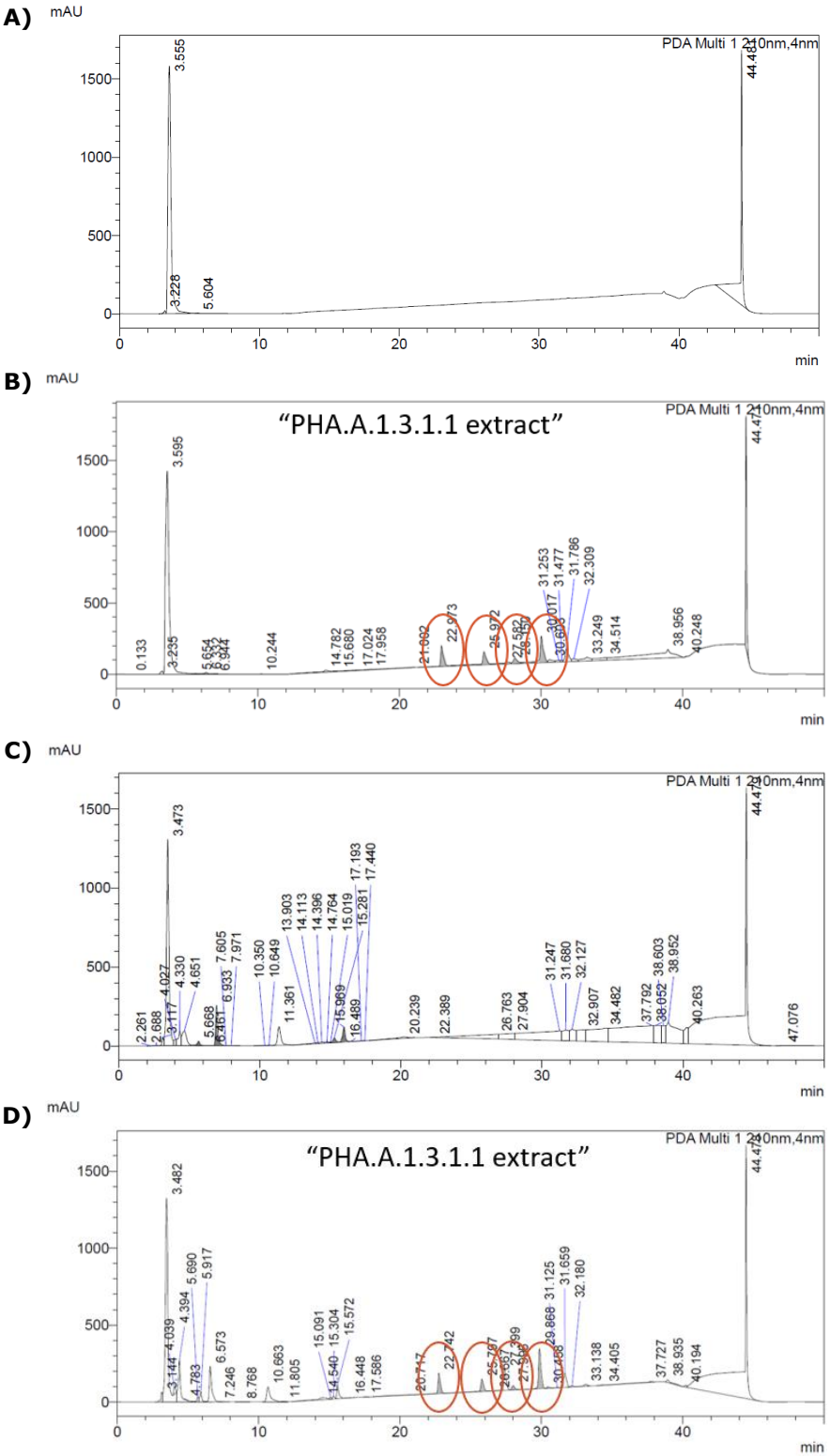

Figure 9 HPLC chromatograms of PHA.A.1.3.1.1 extract in acidic solution (A and B) or cell culture medium (C and D) – comparison between "blank" (just the medium, A and C) and the PHA extract (B and D). The detection was carried out at 210 nm.

In the next phase, the materials developed with these precursors were tested, either to check if the cytotoxic potential remained in the final material (in the case of indigo samples) or if the processing induced harmful / toxic changes in the material properties.

Different samples were tested under ISO 10993-5 (part 12), in similar conditions as the ones used for the precursors, but extracted at 100 mg/mL, and only with perspiration solution (pH 5.5) as this better simulates the contact of the materials with our skin and how it can absorb some substances. The samples tested and main results are presented in Table 5. Figure 10 shows the cell viability for each precursor sample.

We decided that the printed samples (textile and leather) and insole foam were the most critical to test, due to their higher change of having close contact with the skin, therefore, if they had cytotoxic behaviour would be an impediment for their market entrance. As for the plastic films and composites, due to the previous tests on the PHAs and processed PHA, we concluded that further tests would be unnecessary.

None of the materials presented cytotoxic potential, except leather, with cell viabilities always above 85% and no cell lysis was observed, even for the higher concentrations of the extracts. As to the leather substrate, the cytotoxicity observed is most probably due to the leather itself rather than the print, due to the harsh chemicals used during leather tanning, even though the leather used in this project was not chrome tanned.

Table 5 Bio-based materials tested for their cytotoxic potential under ISO 10993-5, and main results

Sample	Extraction media	Result with cells
Foam PHA 20 ShA	Perspiration solution pH 5.5	Discrete intracytopasmatic granules, no cell lysis, **no reduction of cell growth** ✓ No cytotoxic potential!
Indigo 1%_CO_Biopolymer		
Indigo 1%_PES_Biopolymer		
Yellow 0.5%_CO_Biopolymer		
Indigo 1%_leather		Not more than 70% of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50% growth inhibition observable. ✓ Cytotoxic potential!

Figure 10 Evaluation of cell viability of fibroblasts L929 by the MTT test after 24 h incubation with extracts of different bio-based materials (10 mg/mL) in acidic solution, at different concentrations. Differences observed were significant ($p < 0.05$).

2.4. T6.1.4 Ecotoxicity assessment of the materials during degradation

To test the potential environmental toxicity of the developed components, CITEVE, besides carrying out the leaching tests of hazard substances, has assessed the germination success and growth of plants that are relevant in agriculture in substrates containing the degradation products from the biodegradability tests in Task 6.2. This was only carried out in the final stage of the project, as the tests in Task 6.2 were completed, reason why some tests are still in progress.

Results are shown for the germination of *Brassica oleracea* on the substrates after the degradation of the flexible film by PROPAGROUP (33% PHA + 66% PLA) and the foams from NORA. These tests were done following an internal method from CITEVE, based in EN 13432:2015.The substrates from the degradation were used at 50% and 25% mixed with a commercial compost, the germination was carried out at (22 ± 10) °C, (70 ± 25)* relative humidity, and 131 µmol/m2/s of luminosity for 14 days. For the test to have a positive result, the germination rate and plant biomass of the compost samples should be higher than 90% of those of the blank compost (100% commercial compost).

The germination rate is calculated by the formula below:

$$Average\ germination\ (\%) = \frac{Average\ number\ of\ germinated\ seeds\ of\ the\ condition}{Average\ number\ of\ germinated\ seeds\ of\ the\ blank} \times 100$$

The plant biomass is calculated by the formula below:

$$Average\ biomass\ (\%) = \frac{Average\ biomass\ (g)\ of\ the\ condition}{Average\ biomass\ (g)\ of\ the\ blank} \times 100$$

In Figure 11 is a represented an image of the germination after 14 days with the substrate.

*Figure 11 Germination success of Brassica oleracea in substrate from the degradation of **W2BC** materials.*

Both substrates gave excellent results, with germination rates of 98% and 99% in 50% compost from the film and foam, respectively, and average biomass percentages rates of 201% and 211% in 50% compost from the film and foam, respectively.

Currently is ongoing a similar test with substrates from the degradation of Rigid plastic from UDC, flexible film from PROPAGROUP (70%PHA + 30%PLA), PES fabric coated with 2%PHA, and PES fabric control.

3. Conclusions

The materials developed in **W2BC** showed no relevant toxicity in the aspects studied.

The chemical analysis revealed that the hazard substances used in the processes or that could be present in some of the commercial products used revealed that none is present in the materials tested.

The corneometry study conducted allowed us to conclude, with statistical significance, that the tested samples did not cause alterations in the barrier function of the skin's stratum corneum, as a decrease in TEWL values was observed after contact with the skin (negative variations). The positive control (SLS), as expected, caused alterations in the skin barrier function, significantly increasing TEWL values (positive variations) compared to the samples and the negative control (empty occlusion chamber). Regarding the erythema level analysis, it was concluded that the samples did not cause significant changes in erythema levels and, therefore, none of the tested materials represented a visible irritant potential.

The cytotoxicity tests showed that the PHAs (processed and unprocessed), the microparticles, the printed textiles and the PHA foams do not exhibit any cytotoxic potential, with cell viabilities above 85% even for the most concentrated extracts (while the standard considers that a material with a cell viability above 70% does not have cytotoxicity). On the other hand, the printed leather showed some toxicity, which can be due to the harsh chemicals used in leather tanning. Both the commercial and the project produced indigo pigment also exhibited some cytotoxicity, with a cell viability close to 70%. The quinacridone pigment produced by PILI was the most critical one, with very low cell viabilities, possibly due to unidentified impurities or subproducts formed in the process, as the commercial one does not show cytotoxicity.

The germination tests on the substrates from the degradation tests in T62. were a success, with excellent germination success and biomass rates.

These results contributed to the accomplishment of *Milestone 7 – Bio-based components without toxicity*.

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