Application of supercritical carbon dioxide (CO_2SC) to upcycled cosmetic Padouk wood micronized powder microbial decontamination

Keywords: Non-thermal microbial decontamination, micronized powders, natural products, upcycling.

INTRODUCTION

Cosmetic powder decontamination is a challenging issue, particularly in regard of upcycled products which are often highly contaminated. Reaching the microbial bioburden of 1000 or 100 CFU/g for cosmetic products [1] requires an efficient treatment, but powders are adding a supplementary level of complexity. Indeed, many active compounds and flavours are incompatible with thermic treatments. Non-thermal decontamination techniques exist that could solve this problem, but chemical treatments such as ethylene oxide and others, generate trace contaminants difficult to eliminate in powders and other processes, such as ozone or hydrogen peroxide have strong oxidative activity that can affect the integrity of the product [2]. Physical decontamination techniques can be considered although the best known, such as gamma irradiation of electron beam, are not compatible with natural labels, and particularly COSMOS certification, essential for cosmetic products [3]. Softer radiative decontamination techniques, as UV irradiation or pulsed light, perform well on surfaces but are not suitable to powders since the high developed surface of a powder requires excessive energy to preserve efficiency [4]. In addition, energy absorption by powder particles provokes local heating leading to potential explosive reactions and necessitate operating in ATEX-rated environments. Other physical non-thermal techniques, such as non-thermal plasma, have the advantage to be efficient on dispersed powders but remain based on the production of highly reactive oxygen species capable to alter biological molecules [5]. Then, except very high-pressures decontamination techniques, still confidential because of the cost of their environment, all physical decontamination techniques proposed until now are “advanced oxidative processes” [6] and poorly or not adapted to powder treatment.

The first description of supercritical carbon dioxide (CO_2SC) as a decontamination technique dates back to the middle of the 20^{th} century [7] but it remains underdeveloped despite its significant advantages. The first one is the chemical inertness of CO_2SC which does not induce the formation of oxygen reactive species. By the way, it is an ATEX technology and does not require any special protection equipment. The second advantage is that it is not inducing the formation of residues and considering the low quantity necessary for a complete run, it can be flushed in outer air environment at the end of the treatment. The third one that, in its supercritical state CO_2, has remarkable diffusion properties and can diffuse easily in materials and through biological membranes [8]. Then it is considered as a green and GRAS technology. Of course, CO_2SC has also some inherent limitations. Until now it can be only operated by batch, but its major limitation is that its efficiency is variable between microorganisms and uncomplete on spores. In fact, it is not a

sterilization but a decontamination technique. This can be a rebutting default in pharmaceutical industry, but not in cosmetics since except in rare cases, cosmetic compounds are not sterile.

Here we investigated the potential of CO_2SC for the microbial decontamination of micronized padouk wood powder derived from wood chips and barks remaining of wood industry. Traditionally produced in West Africa by simple grinding, padouk wood powder is used by women to treat, protect and colour their hair and skin [9,10] and has potential large applications in cosmetics.

MATERIAL AND METHODS

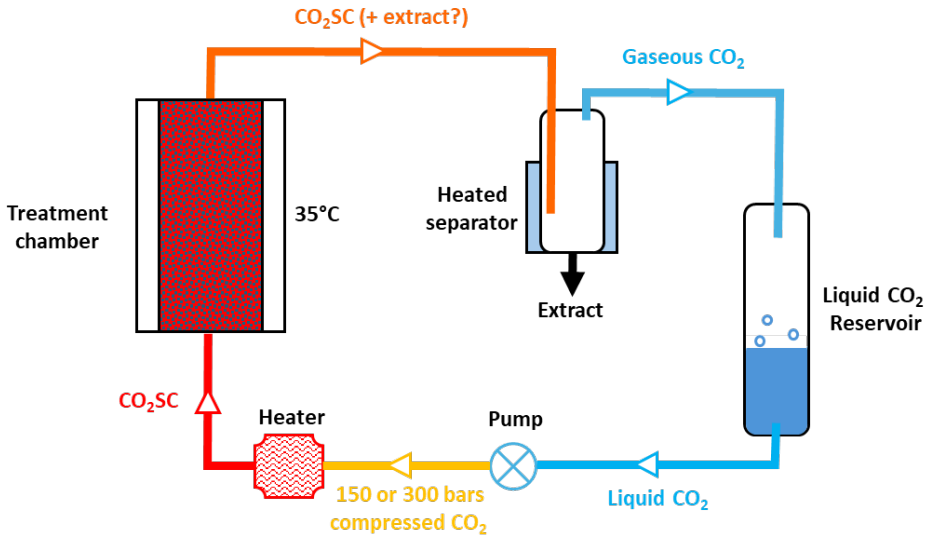
Study material

African padouk (*Pterocarpus soyauxii*) is a large tree (50 m) of dense humid tropical and equatorial forests of the *Fabaceae* family. Abundant and implanted in other tropical regions, it is not submitted to commercial limitations. Its wood has a deep reed to purple-brown colour while the live bark is yellow, a colour which is turning to brown in the outer bark [11]. This wood has high industrial interest because of its durability and is exploited for production of patio planks and garden furniture. It was also traditionally employed as powder by local populations for skin and hair protection and it tinctorial properties have been used since the 19th century [12]. In this study, padouk wood and barks were sourced from cuttings wood wastes collected in an industrial wood exploitation in West Africa (CABD Company, France). After reception, they were dispersed in 1 to 2 cm fragments and wood fragments were subsequently submitted to a micronization step (Naturplast,, France) in order to obtain a 250 µm micronized powder. This granulometry was selected to prevent metal filters clogging in the CO2SC pilot system. Barks were used as a control for potential extractables recovery during CO_2SC decontamination tests, and employed without further treatment.

Supercritical carbon dioxide (CO_2SC) treatment

The CO_2SC pilot system employed in this study is a standard apparatus normally employed for chemical extraction (Model 4356, Separex, France). It is composed of a stainless steel chamber (0.5 L) limited by two metal filters (25 µm) inserted in an incubator compartment adapted to resist high pressures. It receives compressed CO_2 pumped from a reservoir and previously heated to reach the supercritical state at 73,8 bars and 31.1°C between liquid and gas where CO_2 has optimal diffusion properties. After passage through the chamber, CO_2SC is directed to a heated separator where potential extracted materials are separated from CO_2SC. CO_2SC is then condensed to liquid and recycled to the reservoir (***Figure 1***). In this study a first series of test was realized using CO_2SC at 150 bars for 1h at 35°C. Considering the results a second series of experiments was carried out at 300 bars for 3h at 35°C. After treatment and return to atmospheric pressure and room temperature, the steel chamber was immediately removed from the incubator and the powder was transferred into sterile 50 mL Falcon tubes or 250 mL samples boxes.

Figure 1: Schematic representation of a classical CO_2SC pilot



Analytical studies

The bright yellow extract obtained from padouk barks and recovered in isopropanol after CO_2SC treatment was analyzed by mass spectrometry coupled to gas chromatography (GC-MS) using a HP6890 Agilent GC-MS (Agilent, CA, USA) on an Optima-Wax plus and on an Optima-17ms semi-polar capillary column (Macherey Nagël, Germany) with helium as the carrier gas at a column flow rate of 1.2 mL min^{-1}. In both cases, the quadrupole was at 150 °C, and the ion source was at 230 °C. The solvent delay time was 4.0 min. The mass scan range was m/z 10–800.

Microbiological studies

The microbial charge was determined according to the European Pharmacopea 2.6.12 [13] after extraction of the microorganisms by stomaching. For that, 200 mL of a solution of buffered peptone water (BPW) (Biokar Diagnostic, France) at a double concentration (X2, *i.e.* 40 g/mL) was mixed with 2 g of wood powder or barks to neutralise the pH. Then, the suspension was introduced into 400 mL sterile Blender bag lateral filters (VWR, France) and submitted to homogenization for 1 min in a Smasher AESAP1064 stomacher (BioMérieux AES Chemunex, France) followed by 30 min rest at room temperature. The filtrate was then collected under sterile conditions and used for microbial numeration.

Total aerobic bacteria were determined after mass seeding of 1 mL of extract at concentrations ranging from 10^{-1} to 10^{-7} in tryptone soya agar (TSA) (Sigma Aldrich, MO, USA) plating in Petri dishes, followed by incubation for 3 days at 32.5 °C. Total anaerobic bacteria were determined using the same medium and at the same dilution ranges, but after incubation for 3 days at 32.5 °C in a A35 Don Whitley anaerobic workstation (Don Whitley, UK). Determination of the sporulated flora was realized according the ANFOR NF V08-602 guideline [14]. Briefly, samples were submitted to successive thermic shocks by incubation for 10 min at 80°C in a temperature-controlled water bath, followed by immersion in an ice bath until complete cooling of

the tubes. Numeration was realized as previously described by mass seeding in TSA and incubation for 3 days at 32.5 °C in aerobic conditions. Yeast and fungi were numerated according to the ISO 16212 guideline [15] after mass seeding of 1 mL of extract at concentrations ranging from 10^{-1} to 10^{-7} in Sabouraud agar medium (BD Difco, France) followed by incubation for 3 to 5 days at 22.5°C.

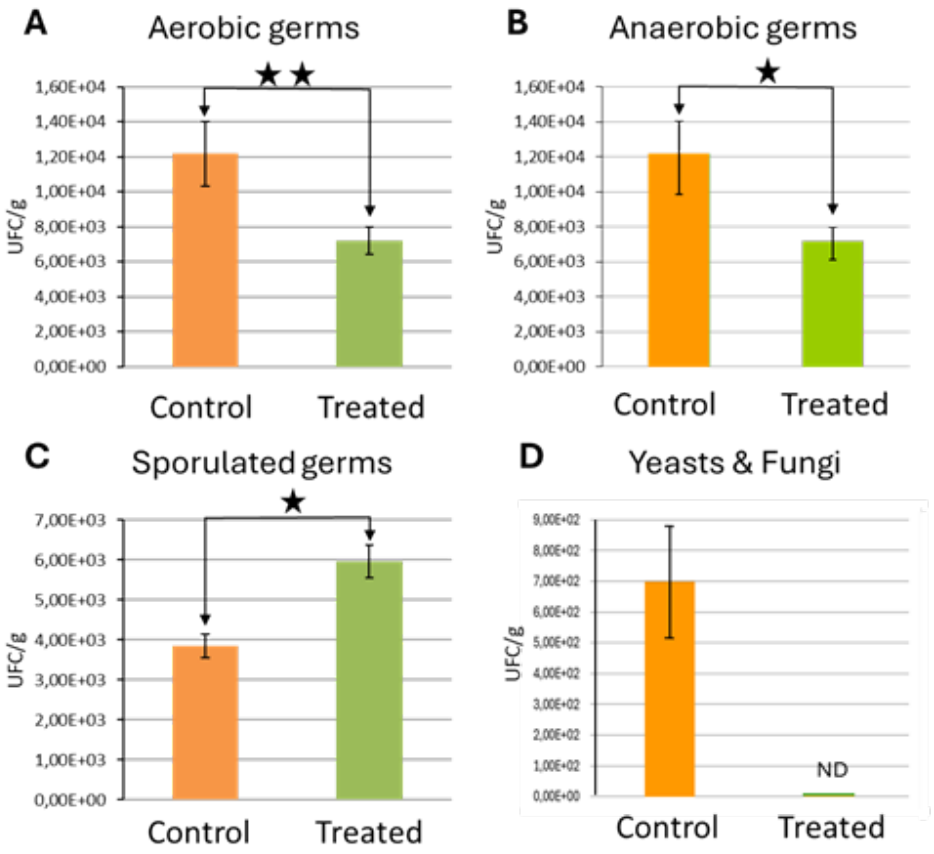
Statistical analysis

Statistical analysis was realized using the open access past3 software for Windows 11 after logarithmic transformation of the data (Log CFU/g). Except the results of yeast and fungi in barcks that were not fitting with a normal distribution law and were evaluated using a non-parametric *Wilcoxon test*, the significance of all other data was tested using the *Students' t-test*.

RESULTS

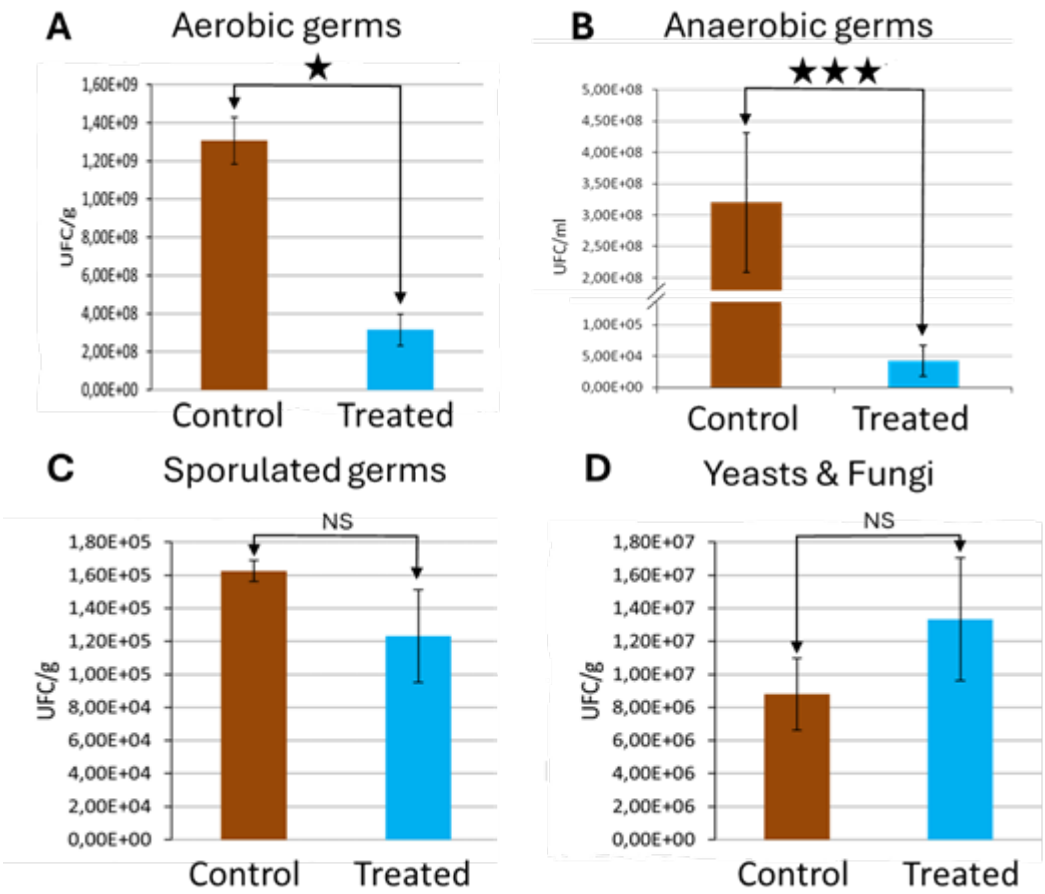
In a first series of experiments, padouk wood powder and barks were treated with CO_2SC at a pressure of 150 bars over 1h at 35°C.

Figure 2: Aerobic germs (**A**), anaerobic germs (**B**), sporulated germs (**C**) and yeasts & fungi (**D**) in control micronized padouk wood powder and after CO_2SC treatment at 150 bars for 1h at 35°C
(* *P*<0.05; ** *P*<0.01; NS: non-significant; ND: not determinable)



In the padouk powder, the initial contamination was low, making reduction statistically difficult to determine. A significant decrease in aerobic germs of 41% (P<0.01). was observed. The decrease in anaerobic germs reached 68% (P<0.05). No reduction of sporulated germs was observed and the total count was even significantly higher after CO_2SC treatment. The reduction of yeast and fungi was not quantified since after treatment the remaining quantity was <10 CFU/g indicating a reduction higher than 99% (***Figure 2***). Although CO_2SC treatment (35°C/1h) was leading to a significant reduction of anaerobic microorganisms, yeast and fungi, the bioburden of the powder remained over the limit of 1000 CFU/g required for cosmetic products. A very limited amount of a yellow extract was recovered in isopropanol after rinsing of the separator suggesting that the padouk wood micronized powder was not containing significant amounts of CO_2SC extractable substances.

Figure 3: Aerobic germs (**A**), anaerobic germs (**B**), sporulated germs (**C**) and yeasts & fungi (**D**) in control padouk barks and after CO_2SC treatment at 150 bars for 1h at 35°C
(* P<0.05; *** P<0.001; NS: non-significant)

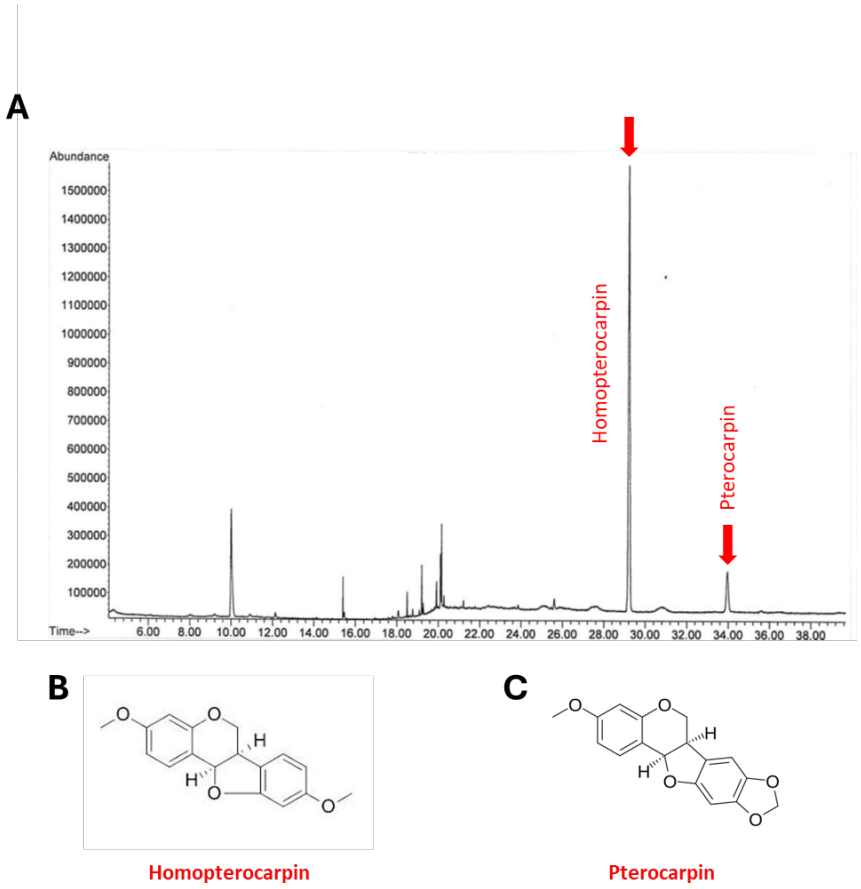


At the opposite of the powder, in barks the initial contamination was very high. A significant 76% reduction of aerobic germs was obtained after CO_2SC treatment (P<0.05). For total anaerobic

germs, the reduction was even higher reaching 99.9% (4.1 Logs) (P<0.001). However, as observed with the powder, no significant reduction of sporulated germs was noted. Additionnally, no change in yeast and fungi contamination was observed, although it should be noticed that in regard of the wood powder, the fungi bioburden of the barks was very high (***Figure 3***). After CO_2SC treatment, a large amount of the same yellow extract was recovered in isopropanol after rinsing of the separator. The yellow color was identical to that the peripheral live bark of the padouk wood.

This extract was analyzed by GC-MS. No detectable compound was observed after 40 min analysis using the polar column. Conversely, analysis on the semi-polar column allowed to resolve a series of peaks and particularly two at 29.237 min and at 33.976 min. These peaks were identified using the internal library of the chromatograph (NIST) as homopterocarpin for first one major and pterocarpin, for the second one. (***Figure 4***).

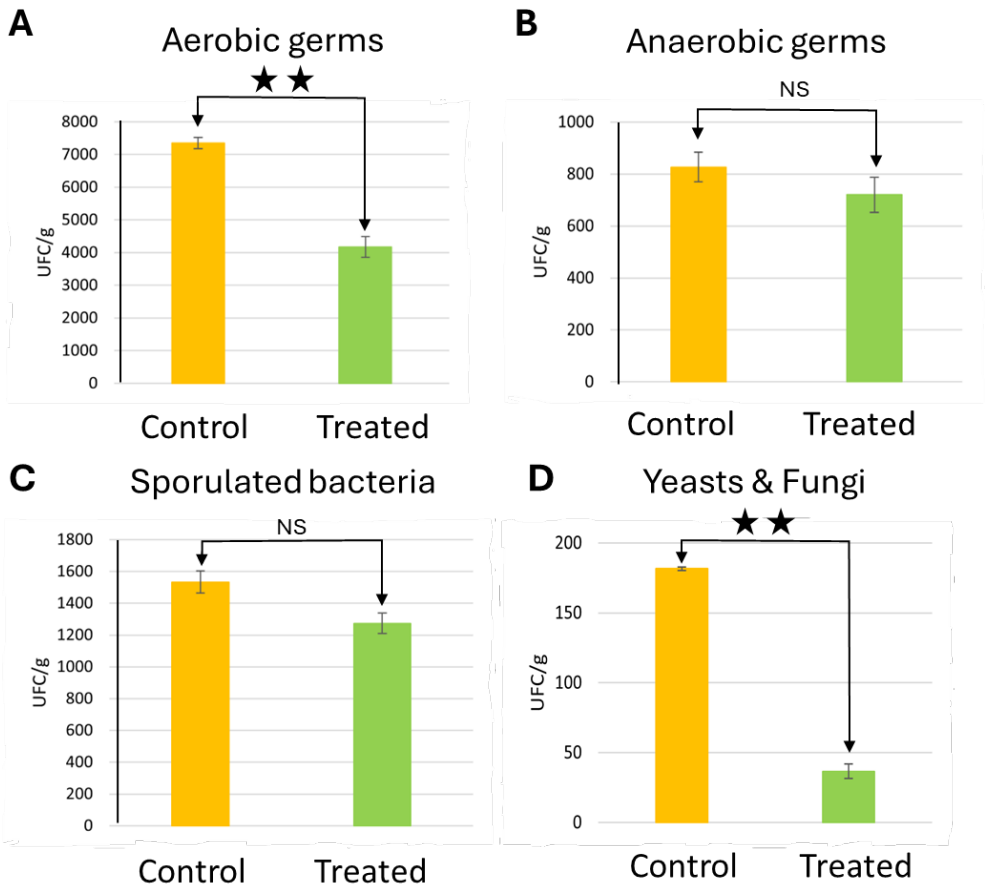
Figure 4: CPG chromatogram showing the peaks resolved from padouk bark using the 17MS Optima semi-polar column (**A**) and the two identified molecules homopterocarpin (**B**) and pterocarpin (**C**)



Preliminary tests having shown that increasing the pressure and duration of the CO_2SC treatment could improve the decontamination efficiency, a second series of experiments was realized on padouk wood powder by exposure to CO_2SC at a pressure of 300 bars over 3h at 35°C. Even after such treatment, the aspect, fluidity, color and granulometry of the powder remained

apparently unchanged. Reductions of the aerobic, anaerobic and sporulated germs as well as yeasts and fungi of 44, 13, 17 and 80 % respectively were noted, but only the decrease in aerobic germs and yeasts and fungi was significant (P<0.005). (***Figure 5***). The bioburden of anaerobic germs in this experiment was too low in this study to can measure a significant reduction. Nevertheless, the final calculated bioburden remained over the limit of 1000 CFU/g for aerobic (3866 CFU/g) and sporulated germs (1273 CFU/g) indicating that it should be necessary to improve this CO_2SC decontamination technique.

Figure 5: Aerobic germs (**A**), anaerobic germs (**B**), sporulated germs (**C**) and yeasts & fungi (**D**) in control micronized padouk wood powder and after CO_2SC treatment at 300 bars for 3h at 35°C
(** P<0.01; NS: non-significant)



DISCUSSION

CO_2SC demonstrates a real efficacy for powder decontamination with results close to, but over, the expected contamination levels for cosmetic products. The lack of efficiency of CO_2SC on sporulated microorganism is consistent with previous studies [16]. In the case of aerobic microorganisms, the limitations are probably due to the high bioburden. Then, it should be necessary to adapt this decontamination technique to make it should more aggressive, albeit it is also essential to preserve the physical and chemical properties of the micronized power.

Mechanisms involved in the decontamination efficiency of CO_2SC are not completely known. As proposed by Ribeiro *et al.* [8] it is postulated that, because of its high diffusion potential CO_2SC is rapidly spreading in materials and solubilizes in the bacterial microenvironment. It is then directly affecting the bacterial membrane integrity, reducing its barrier efficiency. CO_2 is subsequently diffusing into the bacterial cytoplasm where it reacts with water and generates HCO_3^-, which are recycled by bacterial membrane transporters, and H^+ leading to a massive intracellular acidification. This pH decrease is leading to enzymes inactivation and metabolism disorders resulting in disorders of the cellular electrolyte balance, loss of membrane integrity, extraction of cytoplasmic components and finally bacterial death. The lack of efficiency of CO_2SC on sporulated germs should be related to their dormancy, reduced metabolic activity and thick outer wall capable to limit CO_2 diffusion [17]. Different strategies have been tested to enhance CO_2 diffusion through the spore capsule including addition of co-solvents (ethanol, acetic acid, hydrogen peroxide…), but with variable improvements in decontamination efficiency [18, 19]. One of the more effective and inert solution tested was introduction of purified water into CO_2SC, since water is softening the capsule, leading to an increase of CO_2 penetration [20]. However, this technique is not suitable to micronized powder, since water can lead to particle agglomeration [21]. Another solution should be to reproduce the spore activation technique used for numeration before CO_2SC treatment postulating that revivified microorganisms should be more sensitive to CO_2. This technique is simple and consists in a thermal shock realized by incubating the samples at 80°C before a rapid cooling in ice. However, increasing the temperature to 80°C is not compatible with heat sensitive materials and non-thermal decontamination. Nonetheless, this strategy of thermal shock can be run in another sense, by decreasing the temperature and allowing the sample to return rapidly to 35°C as required to keep CO_2 in its supercritical state. Such strategy will be now operated in a third study by immersing the sample in liquid nitrogen (-196°C), which is also chemically inert, before mounting the sample in the treatment chamber an injecting CO_2SC at 35°C.

Another adaptation of the system due to the fact that it will be necessary to solve the question of the extraction potential of CO_2SC. Indeed, CO_2SC is a well-established extraction technique and in the present case we observed it could extract active molecules such as homopterocarpin and pterocarpin from padouk bark, and probably in a lower extend from the wood powder. Both molecules have valuable potential applications. Homopterocarpin (CAS 606-91-7) is an isoflavonoid with antioxidant properties [22]. It has also antifungic, insecticide and antimitotic activities |23]. Pterocarpin (CAS 524-97-0) is coumarochroman capable to inhibit bacteria and yeasts (*Candida* sp.) development [24]. These two molecules are probably essential in the hair and skin protective activities of padouk powder as used traditionally in Africa. Then, they should be preserved into the product during CO_2SC decontamination. Since it is impossible to limit the extraction and diffusion potential of CO_2SC, the solution should be to modify the circuit in order to

re-inject extracted molecules into the product in a closed loop. This modification of the classical CO_2SC device should be easy to realize, but essential to make optimal the CO_2SC decontamination technology.

Operating by batch, the industrial scalup of CO_2SC is ready with devices capable to treat more than 450 kg. Standard CO_2SC pilots, as used in this study, are not sufficient to reach the limit of 1000 CFU/g as necessary for cosmetics, but solutions exist to improve this technique that should be the first fully adapted solution for cosmetic powders microbial decontamination.

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