SSD

SCIENCE FOR A SUSTAINABLE DEVELOPMENT

DEVELOPMENT OF A NEW LOW-COST AND REGENERABLE DETECTION DEVICE FOR MICROBIAL COMPOUNDS

MIC-ATR

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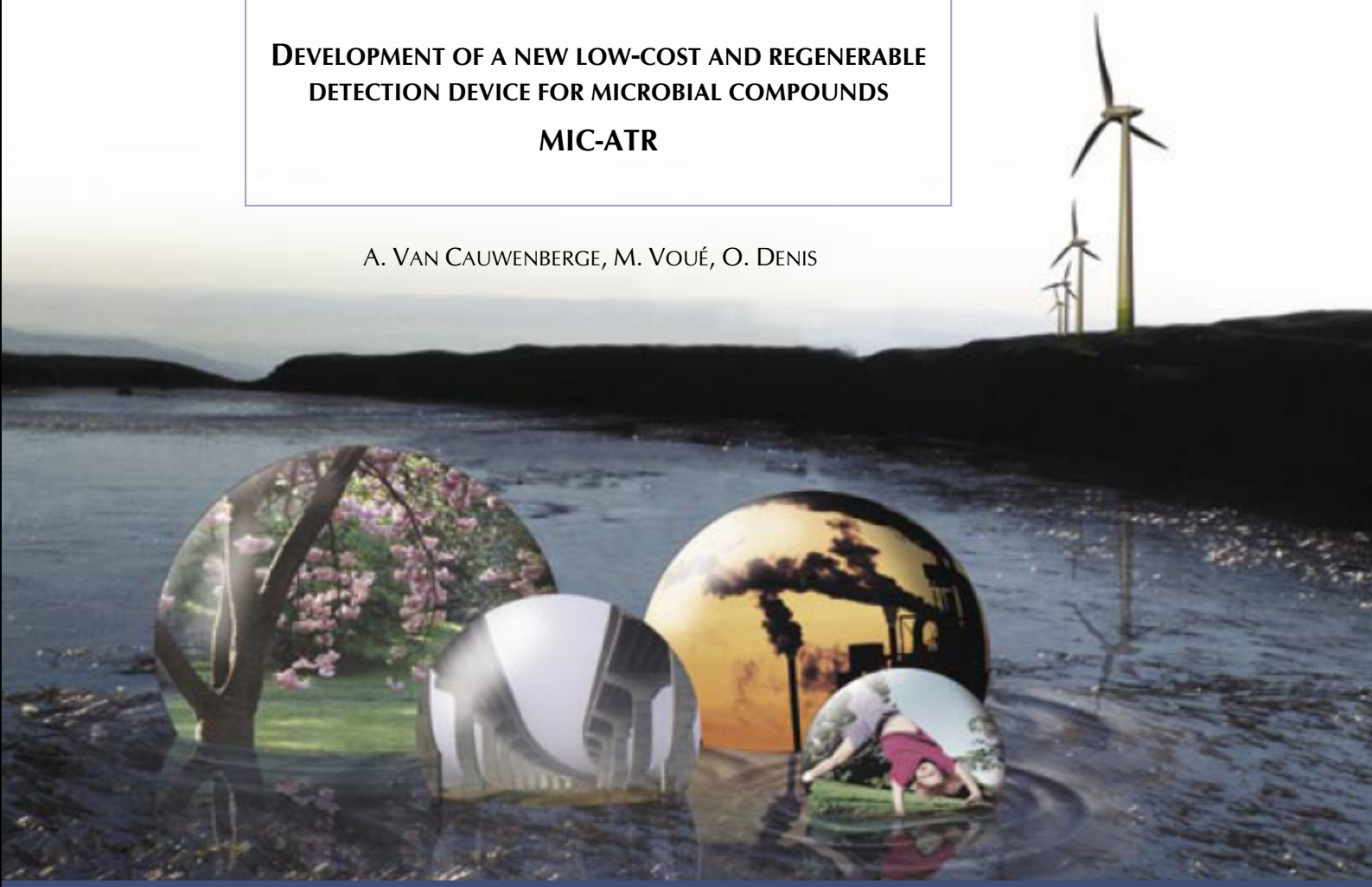

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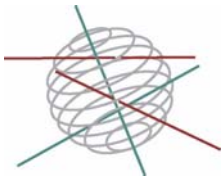

Health & Environment

FINAL REPORT PHASE 1
SUMMARY

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A. Van Cauwenberge, M. Voué, O. Denis "***Development of a new low-cost and regenerable detection device for microbial compounds MIC-ATR"*** Final Report Phase 1 Summary Brussels : Belgian Science Policy 2009 – 6 p. (Research Programme Science for a Sustainable Development)

SUMMARY

There is crucial concern about the presence of molds in indoor environments and their adverse effects on human health. The indoor molds, omnipresent in 60% of the dwellings, have indeed the potential to produce components that have been associated to several severe human health problems like allergic hypersensitivity responses, symptoms of asthma, pulmonary haemorrhage, potentially mortal. For instance, in Belgium, according to the Scientific Institute of Public Health, the prevalence for asthma is about 4% in the global population and is relatively stable between 2001 and 2004.

Fungal spores are universal atmospheric components and are recognized as important causes of respiratory allergies. Fungi grow on most substrates if enough moisture is available, frequently colonize indoor damp places and their spores are commonly found in house dust. More than 80 genera of fungi have been associated with symptoms of respiratory allergy. Among the various mold species Alternaria, Cladosporium, Penicillium and Aspergillus are frequently considered to be important causes of allergic rhinitis.

Four fungal components have been identified as components of interest: VOCs, fungal spores, airborne mycelium fragments, mainly containing glycan wall fragments and mycotoxins, which are non - or weakly volatile stable secondary metabolites.

The links between the presence in the environment of these compounds of interest and identified and declared pathologies is, most of the time, indirect. Among the identified causes of asthma, living in poor indoor environment has often been highlighted. In such kind of environment, dampness is the principal factor of development of mold. Visual inspection doesn't allow to fully asses any adverse health effect. The risk associated to mold should be characterised by the presence of mycotoxins in ambient air. The indoor molds have indeed the potential to produce extremely dangerous toxins. Exposure to these factors has been associated to the several severe human health problems cited above. The most dangerous mycotoxins responsible for these belong to the family of aflatoxins and trichothecenes.

Mycotoxins have been intensively studied in the context of food safety. Mycotoxins, by-products of fungal metabolism, have been implicated as causative agents of adverse health effects in humans and animals that have consumed fungus-infected agricultural products. In this context, the link between the amount of toxin and the observed pathology is more direct. In such a way, normalization actions were carried out, defining the upper admitted levels of such compounds in foodstuffs. These limits are not defined for airborne mycotoxins, due to the lack of experimental data and the absence of reliable sampling and testing procedures.

To date, studies have mostly focused on detecting mycotoxins on bulk materials or in settle dust but there is an urgent need, driven by the guidelines of Public Health policy, to develop specific and sensitive tests to measure airborne macrocyclic trichothecenes mycotoxins in indoor environments, for which no specific nor enough sensitive detection method exists.

Therefore, we propose to overcome these drawbacks by developing a regenerable low-cost biosensor of high sensitivity and selectivity based on FTIR/ATR spectroscopy and to use it to monitor the ligand/receptor interactions of these molecules. The biosensor uses optical elements, transparent in the IR spectral domain, modified by wet chemistry to allow the coupling of molecular receptors.

A Nicolet 380 FTIR spectrometer has been successfully installed at HVS location. The FTIR-sensor experimental cell consists in a vertical ATR SPECAC flow cell connected to a Watson-Marlow peristaltic pump achieving flow rates from 5 to 50 µl/min. The FTIR elements are trapezoidal germanium crystals (50 x 20 x 2 mm³, angle: 45°) that are polished and functionalized at the CRMM. The system has been successfully qualified with two types of FTIR experiments, the determination of the percentage of ethanol in water solutions and the binding of biotin on avidin FTIR sensor.
The equipment was then able to produce experimental results on more sophisticated systems, as we showed with the detection of low molecular weight molecule like DNP which serves as model for detection of haptens. Results obtained by "classical" competitive ELISA and by FTIR were compared, with the use of 6 different rat monoclonal antibodies specific for the DNP. All the tested antibodies responded in a similar manner to the coupled DNP molecules (DNP-HSA) but significant differences were observed for the recognition of free DNP molecules. With coupled DNP molecules, the limits of detection were equivalent between both techniques: in the range of 5-15 ng/ml for the FTIR assays and about 40 ng/ml for the ELISA method, but for the free DNP molecules, the limits of detection were different: 1 µg/ml with ELISA and 4 ng/ml with the FTIR assays using the LO-DNP34 antibody, which is a level comparable to the one obtained with coupled molecules.

The workpackage 2 was dedicated to the detection of aflatoxin B1. Preliminary experiments were run to monitor the binding of an anti-aflatoxin B1 antibody on a functionalized germanium crystal. The molecular layers are equivalent to what has been used for DNP detection. Using a competitive ELISA we tried to determine the concentrations of aflatoxin B1 in our environmental samples. We analysed 36 environmental samples from our collection; 15 of these were previously found to be positive in mass spectometry for the presence of aflatoxin B1. Due to the lower sensitivity of our ELISA assay as compared to the mass spectometry, we were unable to confirm the presence of the toxin in our samples. It should be noted that this lack of sensitivity of optical methods such as ELISA tests can be partially compensated by an appropriated choice of a spectral method for which a dedicated spectral range can be selected to monitor a specific binding. Further experiment to detect the toxin will be carried out during phase 2 of the project.
As molds are very common outdoor but are also present indoor in damp places, quantification of the mold biomass in the ambient air turned out to be interesting in order to better appreciate the level of indoor pollution. Therefore, an additional workpackage has been added, in order to produce and characterise monoclonal antibodies against Alternaria, Aspergillus and Stachybotrys spore fragments.
In order to obtain rat MoAb directed against components of the mold, LOU/c rats were immunized in the foodpats with 5.10^6 spores of Alternaria alternata (IHEM 18586) or Aspergillus fumigatus (IHEM 6117) or Stachybotrys chartratum (IHEM 22013). At the end of the immunization, lymphocytes were obtained from the poplietal lymph nodes. Lymphocytes were fused with the IR-983F cells. Growing hybridomas were selected in HAT medium. Positive clones were selected by fluorocytometry on various mold spores. Five MoAb were selected from the rats immunized with Alternaria alternata and characterised. LO-ALT-3 was shown to be clearly species specific while LO-ALT 5 seems very interesting to detect a large array of mould species in environmental samples. We analysed the ability of LO-ALT-5 MOAb to detect the antigen in environmental samples, coming from dust vacuumed on one hundred centimetres

square surface from ten different living rooms. The ELISA with LO-ALT-5 was able to detect the presence of an antigen in four out of ten samples. Six other MoAbs have been selected from rats immunized with Aspergillus. From these six antibodies LO-ASP-2 demonstrated a very good binding on Aspergillus niger spores and a low binding to Alternaria, Cladosporium and Stachybotrys spores. Five others MoAbs have been obtained from rats immunized with Stachybotrys. These antibodies need to be better characterized before being used in our biosensor.
The anti-Alternaria MoAbs were successfully immobilized at the surface of the optical sensor and their spectral response monitored, as a function of the dilution of the Alternaria extract, in the polysaccharide and in the protein spectral domains. Preliminary results show that the antibodies mainly respond to the polysaccharide part of the antigen.

The workpackage 3 was dedicated to the production of monoclonal antibodies against mycotoxins. As mycotoxins are small non proteinic components, they are not able to induce the production of antibodies when injected "as this" in animals since the production of antibodies (at least for non repetitive antigens) requires the help of T helper cells recognizing linear peptides. Indeed the activation of B cells, their production of antibodies and the generation of MoAb upon immunization requires the conjugation of the toxins to carrier proteins.

Therefore Roridin A and Verrucarin A were conjugated to the KLH and OVA. Since these toxins do not have a functional group to facilitate their conjugations, they were treated with succinic anyhydride to generate bis-hemisuccinate. An activated N-hydroxysuccinimide intermediate of the toxins-hemisuccinate was synthesized. Protein toxins conjugates were then prepared by coupling the activated ester reactive intermediate to the carrier's proteins using N,N′-Dicyclohexylcarbodiimide.
LOU/c rats were immunized in the foodpats with 50 µg of roridin A or verrucarin A conjugated to the KLH. At the end of the immunizations, lymphocytes were obtained from the poplietal lymph nodes. Lymphocytes were fused with the IR-983F cells. Growing hybridomas were selected in HAT medium.

The efficiency of the immunization/fusion was confirmed by the presence of numerous clones specific for the KLH. More than 300 clones have been obtained and analysed. More than 60% of these clones were specific for the KLH but none were directed against the mycotoxins.

Therefore we decided to use another coupling reaction. We still generated a bis-hemisuccinate of the roridin A or the verrucarin A and these products were immediately coupled to the proteins (BSA, OVA or KLH) using a water soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride).
LOU/c rats were immunized in the foodpats with 50 µg of verrucarin A conjugated to the BSA. Poplietal lymph node cells were fused to IR-983F cells. Growing hybridomas were selected in HAT medium and their supernatants were tested by ELISA on plated coated with BSA (to monitor for the secretion of antibodies specific to the carrier) or plates coated with BSA-Verrucarin A to detect mycotoxin specific antibodies.
Eight different MoAb recognizing the mycotoxins were obtained. Supernatants from some of these clones contained antibodies recognizing verrucarin A in the context of different carriers (BSA, OVA and KLH). Some antibodies were also cross-reactive against the roridin A. We investigated the fine specificities of these MoAb but the results

indicated that our antibodies were only detecting the coupled toxin (in a proteinic context) but not the free toxin, therefore impending further development of our detection assays. Therefore we started new fusion experiments with LOU/c rats immunized in the foodpats with 50 µg of verrucarin A conjugated BSA. Poplietal lymph node cells were fused to IR-983F cells. Growing hybridomas were selected in HAT medium and their supernatants were tested by ELISA on plated coated with OVA-Verrucarin A in the absence or in the presence of 10 µg/ml of free verrucarin A (to directly monitor the binding of antibodies to the free verrucarin A). Of the 553 tested clones, 70 clones (13%) produced antibodies recognizing the verrucarin A bound to OVA. Only one of these clones produced antibodies which were inhibited by the free verrucarin A. After optimization of a competitive ELISA test using this antibody (F24-1G2), we obtained sensitivity between 3.9 and 1.9 ng/ml of free verrucarin A. In the next step we will use this assay in order to detect the verrucarin A in our environmental samples.

About 40 samplings were carried out in contaminated houses during the first semester 2008. The airborne toxins were collected on quartz filters (pore diameter: 2.2 µm) by sucking an air volume corresponding to that of ½ of the space at a flow rate of 400 L/min. In parallel to the development of our analytical tools, a cross-validation was requested at the University of Ghent (Prof. S. De Saeger) who analyzed the samples by LC-MS-MS.
In 15 of these 40 samples, mycotoxins were readily detected as concentration statistically significant. Unexpectedly, Aflatoxin B1 was also found, shedding a new light on WP2, which becomes for these reasons of great importance.
A comparison was also carried out with the commercial ELISA test Envirologix®. Discrepancies between LC-MS-MS results and ELISA results are important and the former seems to systematically under-evaluate the contamination.
Concomitantly, about 40 samples were also collected in non-contaminated houses ("blank"), following the sampling procedure previously described, in order to make comparisons and build the first step of an epidemiologic study campaign that could be carried out in a forthcoming project.

In conclusion, the **technology transfer** between UMons and HVS successfully occurred. On the **DNP model system**, an inhibition optimized ELISA test has been set-up, with a comparison between free DNP and DNP coupled to albumin. Immuno-assays concepts have been successfully transferred to FTIR sensors domain, yielding a new sensors category: FTIR immuno-sensors. **Monoclonal antibodies against Alternaria, Aspergillus and Stachybotrys were produced and partially characterized.** In particular, we have developed and characterized two antibodies (LO-ALT-1 and LO-ALT-5) specific for an antigen present at the surface of mould spores and another antibody (LO-ALT-3) recognizing an antigen specific for Alternaria alternata. We have started to produce and purify these antibodies in order to use them in our biosensor device to quantify the mould biomass indoor. **The production of MoAbs against mycotoxins is ongoing.** Five cross-reactive rats MoAb are recognizing both the roridin A and the verrucarin A but one antibody, the F24-1G2 allows the detection of free verrucarin A with a sensitivity between 3.9 and 1.9 ng/ml.
Environmental sampling started Jan. 2008 and is still ongoing. Cross-validation has been carried out with LC-MS-MS technique, as well as with ELISA (Envirologix®). The latter technique seems to systematically under-evaluated the contamination level.