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Use of reliable bioassays to detect potential hazard of food contact materials extracts to ensure quality, safety and innovation of paperboards

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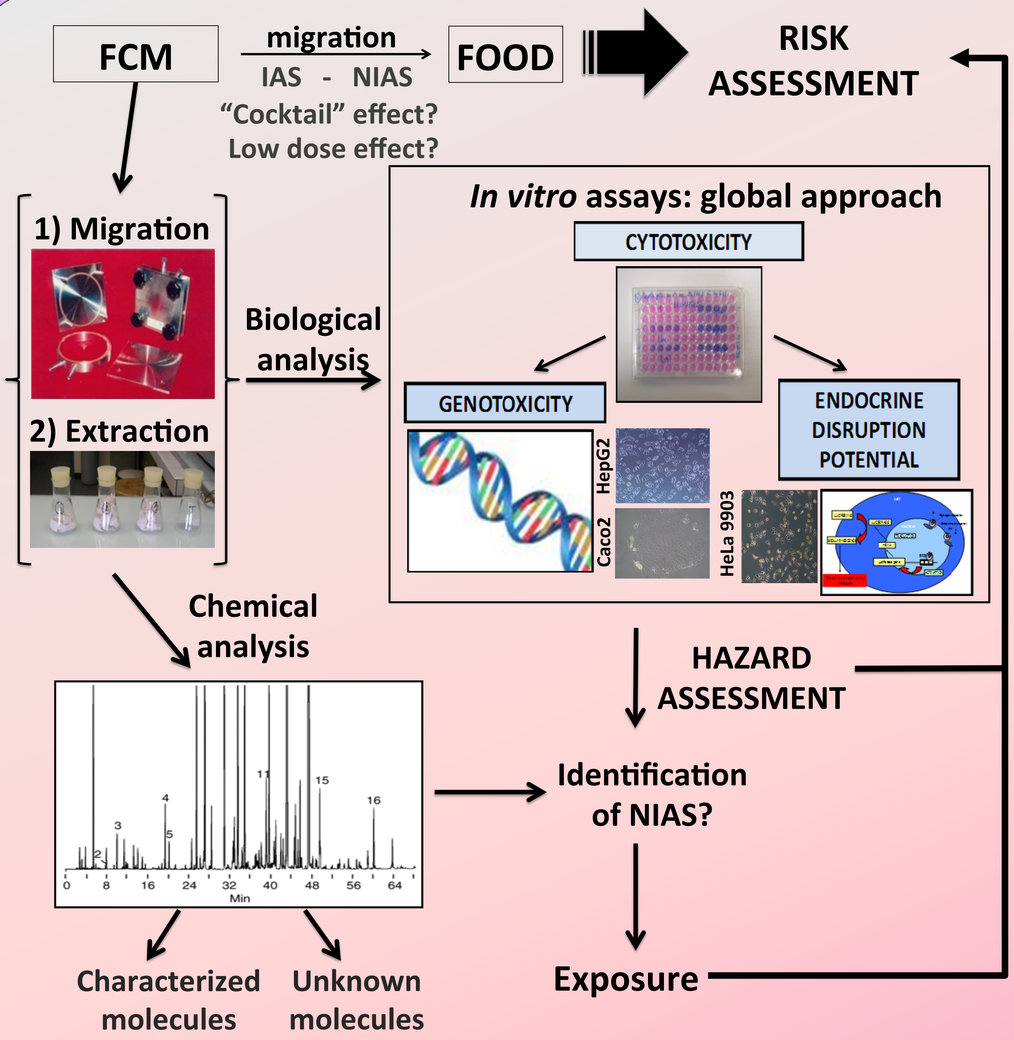
Food contact materials (FCM) represent a major economic issue and also a field of innovation. Food packaging production must meet manufacturing good practices, safety for human health and thus be in compliance with 1935/2004 european regulations. Its third article specifies that FCM do not transfer their constituents to food in quantities which could endanger human health under normal or foreseeable conditions of use. Indeed, these materials are not inert and, in addition to started substances, non-intentionnally added substances (NIAS) are able to migrate from FCM into food. NIAS can be contaminants from recycled materials, impurities, new substances occured during the packaging production chain, synthetic residues etc., and they could represent great part of migrating substances (Grob et al., 2006 ; Skejevrak et al., 2005). The European Regulation No 10/2011 on plastic materials, multilayers and articles intended to come into contact with food is the first Commission regulation that regulates NIAS, but there are no available guidelines to assess the risk of NIAS. Furthermore, NIAS may also be present in other materials, such as paper and boards. Because of the complex nature of FCM, it is pertinent to evaluate the potential toxicity of FCM as a whole and then to take into account any « cocktail effect ». The objectives of this study are to provide to packaging and food companies scientific relevant tools combining physicochemical and toxicological strategies based on bioassays applied to FCM extracts, especially paper and boards. Three toxicological endpoints must be checked as relevant for low dose exposure : cytotoxicity, genotoxicity and endocrine disruption.



Application to paper and boards FCM

Evaluation of *in vitro* toxicity and identification of the critical stations along the production chain of a packaging industry.

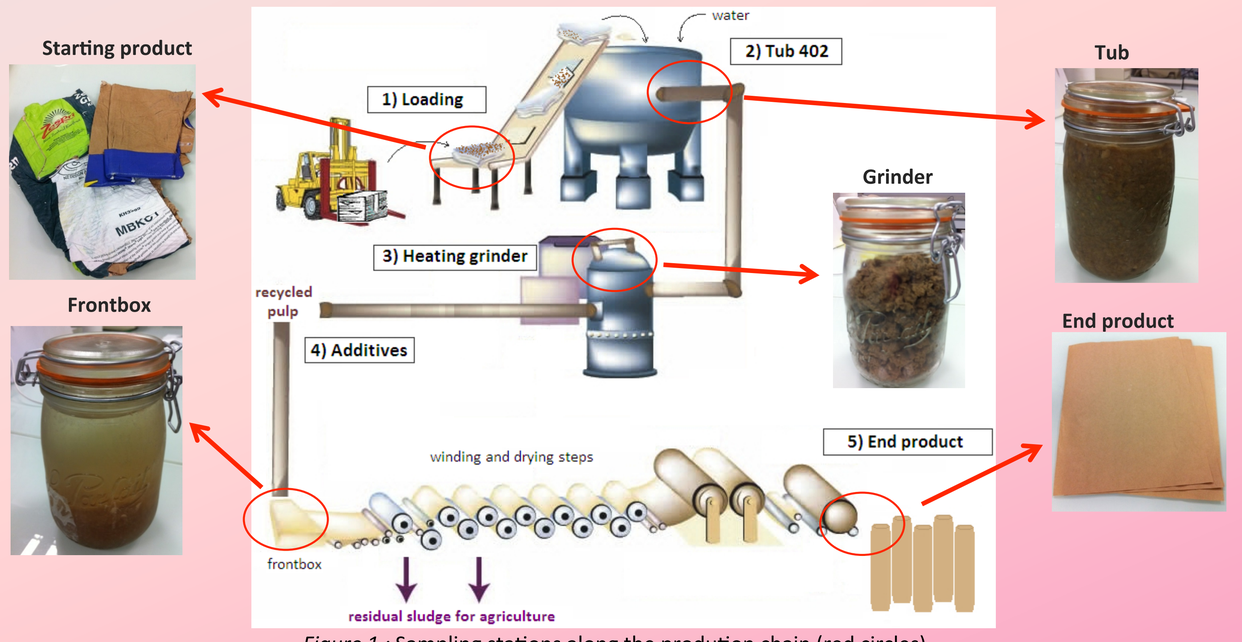


Figure 1 : Sampling stations along the prodution chain (red circles).

Protocol of extraction

	Samples	Preservation before extraction	Direct extraction	Preservation after extraction	Before using
Aqeous form	**Tub** (c° = 1,5g/mL)	4°C	1) centri. 10^3g-30min. 2) recovery of supernatant	- 20°C (glass flacons)	Sterilization by filtration
	Frontbox (FB)(c° = 1,5g/mL)				
Non aqeous form	**Grinder** (c°=1,5/5 g/mL)	4°C	1) small squares 2x1cm. 2) addition of water. 3) orbital shaking 24h - 23°C. 4) recovery of extracted water	-20°C (glass flacons)	Sterilization by filtration
	Starting product (S.P.) (c°=1,5/8 g/mL)	Room temperature in aluminium foil			
	End product (E.P.) (c°=1,5/10 g/mL)				
Norm	**End product (Norm)** (c°= 0,04g/mL)	Room temperature in aluminium foil	Norm AFNOR NF EN 645 (c°= 10g / 250mL)	-20°C (glass flacons)	Sterilization by filtration

Cytotoxicity assay

Resazurin assay

- **Sensitive bioassay to quantify cell viability.**

Principle: Colorimetric and fluorimetric assay based on metabolization of resazurine by mitochondria in viable cells.

➢ **T +** → dodecylsulfate sodium SDS 3%

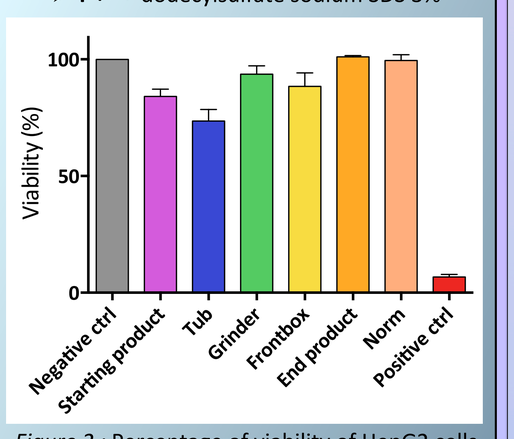


Figure 2 : Percentage of viability of HepG2 cells after 20 h exposure to all extracts of the production chain ; three independent experiments.

Decrease of cell viability after treatment with starting product and tub, but no cytotoxic effect of other samples, in comparison to negative control.

Genotoxicity assays (1)

Comet assay

- **Sensitive bioassay detecting and quantifying DNA primary damages.**

Principle : Detecting single and double strand breaks of DNA, and alkali-labile sites by electrophoresis in alkaline condition (pH = 13) after staining of DNA with a fluorescent intercalating agent [2] (propidium iodide)[3]

➢ **T+** → Metyl methanesulfonate $C_2H_6O_3S$ (50 µM)

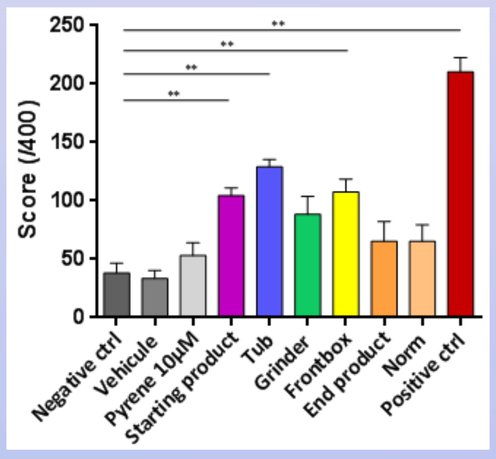
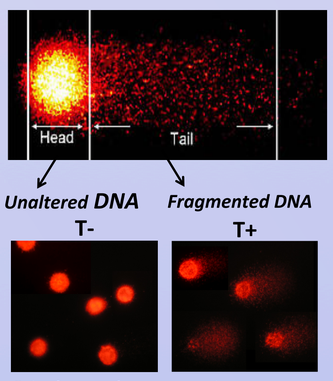


Figure 3 : Genotoxicity evaluation on HepG2 cells after 20h exposure to all extracts of the production chain. Three independent experiments ; two-two comparison of samples with Wilcoxon-Mann Whitney test, ** p<0,01



2 independent experiments were performed and the results were averaged and expressed as a score. Formula score = (comets class 0 x 0) + (comets class 1 x 1) + (comets class 2 x 2) + (comets class 3 x 3) + (comets class 4 x 4).

Significant increase of DNA damages of samples of the production chain in comparison to negative control but no genotoxicity at the end of the process.

Micronucleus assay

- **Sensitive bioassay to detect aneugenic and clastogenic activity of tested substances.**

Principle : Detecting presence of micronucleus in cells having made a mitosis and blocked in anaphase because of the adjonction of Cytochalasine B. Micronuclei can be due to acentric chromosomes or chromosomes not capable of migrate to cell poles.

➢ **T+** → Vinblastine (10 µM)

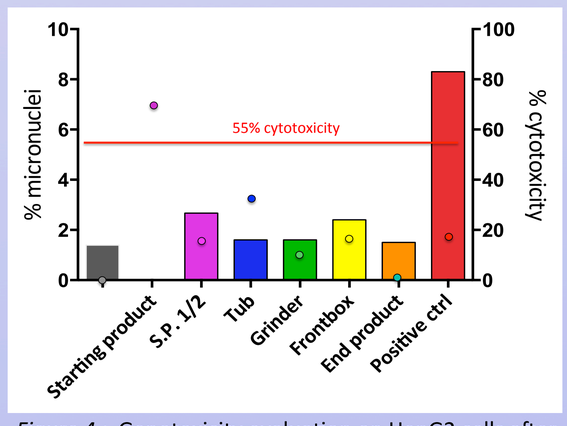
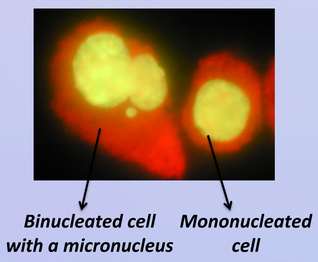


Figure 4 : Genotoxicity evaluation on HepG2 cells after long time exposure to all extracts of the production chain. One experiment.
S.P. 1/2 (starting product two fold diluted) analysed because of the high cytotoxicity of starting product (69% cytotoxicity).



Picture of cells stained with orange acrydine and observation of cells with microscope.
1st cell counting -> percentage of cytostase
2nd counting: percentage of micronuclei

No cytotoxic effect (% cytotoxicity > 55%) of samples excepted for the starting product.

No increase of the number of micronuclei in comparison with the negative control.

Genotoxicity Assay (2)

Ames test

- **Reverse mutation assay performed to evaluate potential mutagenic properties of tested substances on a bacterial model (Salmonella Typhimurium).**

Principle : Detect ponctual mutations (substitution, addition, deletion of one or several DNA bases). The growth of strains of S. Typhimurium used is dependent of histidine amino acid (here TA98 and TA100). Spontaneous reversion occurs allowing the revertants to grow on a media without histidine; mutagenic substances cause an increase in the number of revertant colonies relative to the background level.

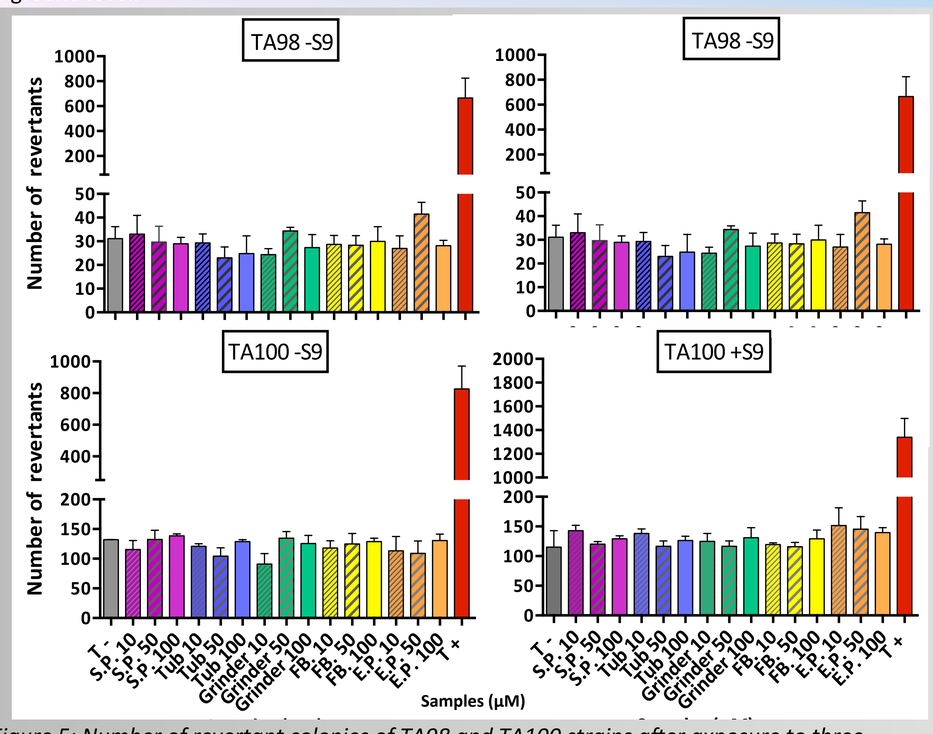
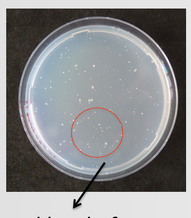
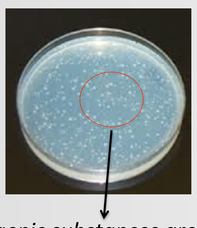


Figure 5: Number of revertant colonies of TA98 and TA100 strains after axposure to three concentrations of samples of the production chain, with or without metabolic activation S9. One experiment performed; three replicates by condition. Positives controls: TA98 without S9 T+: 2-nitrofluorene; TA98 with S9 T+: benzo(a)pyrene; TA100 without S9 T+: azide sodium; TA100 with S9 T +: 2-aminoanthracene



Background level of revertant colonies.



Mutagenic substances are able to cause an increase of the number of revertant colonies relative to the background level.

No mutagenic effect on both strains of all samples of the production chain.

Discussion/Conclusion

Cytotoxicity

- None of the samples were significantly cytotoxic for HepG2 cells.

Genotoxicity

- Ames test: None of the tested samples were significantly mutagenic on both strains of Salmonella Typhimurium used.
- Comet assay: Positive effect detected all along the production chain excepted for the end product.
- Micronucleus assay: Cytotoxic effect observed after long time exposure to starting product but no cytotoxic effect of other samples of the chain. No positive effect of all tested samples regarding the number of micronuclei.

Perspectives

- Positive effect detected with Comet assay and negative effect with micronucleus assay maybe due to DNA reparation.
- Need to establish a dose effect using Comet assay and micronucleus assay.
- Comet FPG could be performed to detect oxidized DNA bases
- Potential oxidizing effect of samples of the production chain could be checked with DCFDA assay.
- Check, using a chemical analysis, which substances could be responsible of the hazardous effects
- Check the endocrine disruption effect as an other toxicological endpoint