Reliable bioassays to detect potential hazard of paperboard food contact material extracts

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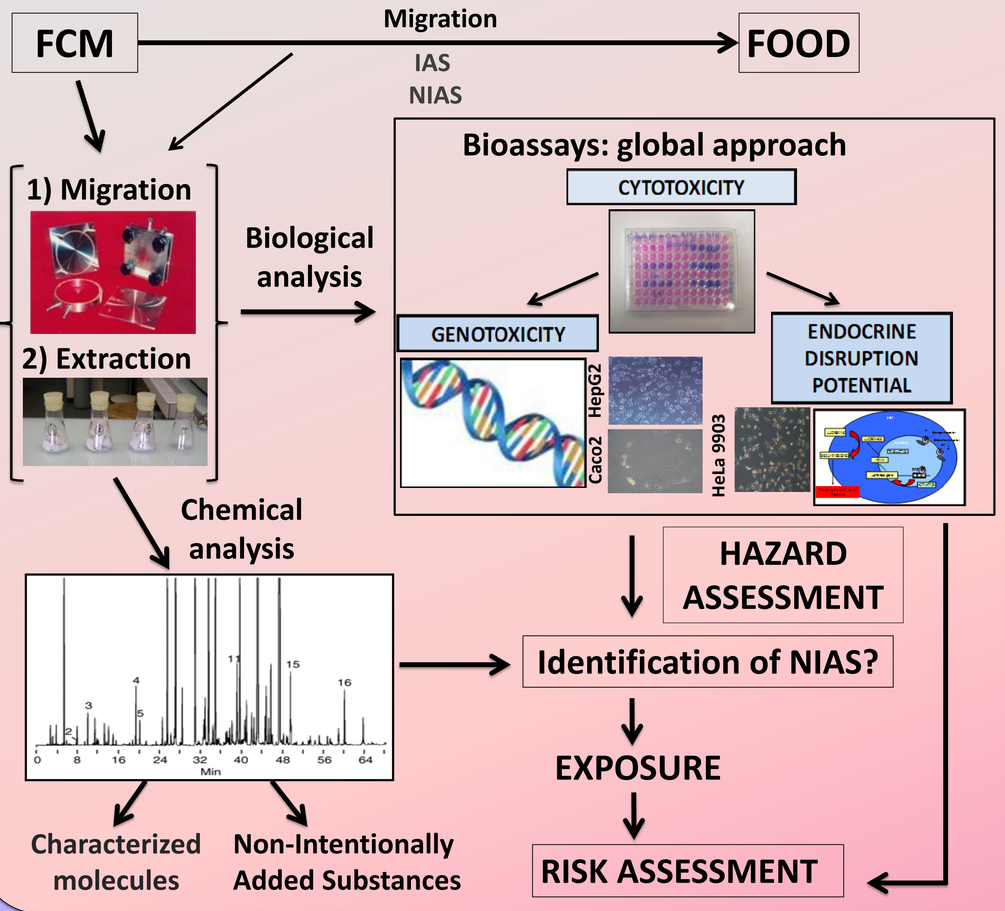
Food contact materials (FCM) represent a major economic issue and a large field of innovation. Food packaging production must be in compliance with the European Regulation 1935/2004: the third article specifies that FCM must not transfer their constituents to food in quantities which could endanger human health under normal or foreseeable conditions of use. Indeed, these materials are not inert and, in addition to started substances, Non-Intentionally Added Substances (NIAS) are able to be released into the food. NIAS can be contaminants from recycled materials, impurities, synthetic residues, new substances formed along the packaging production chain… Then, NIAS could represent a larget part of migrating substances which are not risk assessed, some being unpredictabled (Grob *et al.*, 2006 ; Skejevrak *et al.*, 2005). The European Regulation 10/2011 on plastic materials, multilayers and articles intended to come into contact with food regulates NIAS. It requires that the notion of the risk engendered by a substance concerns the substance itself, the impurities of this substance and any reaction or degradation products that are foreseeable in the context of the intended use. However there are currently no available guidelines to assess the risk of NIAS. They may also be present in other FCM than plastics, such as paper and boards... Because of the complex nature of FCM extract, it is pertinent to evaluate the potential toxicity of FCM as a whole taking 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. Four toxicological endpoints were checked as relevant for low dose exposure: cytotoxicity, genotoxicity , oxidative stress and endocrine disruption activities.

Global strategy for the risk assessment of food contact materials



Evaluation of *in vitro* toxicity and identification of the critical steps along the production chain of FCM paper and boards extracts.

Figure 1 Sampling stations along the prodution chain (red circles), (adapted from lepapier.fr).

Sample preparation

Aqeous form: direct extraction

Sample	Preservation before extraction	Extraction	Lyophilisation and recovery of dry matter content	Preservation after lyophilisation	Before testing
Tub	4°C	1) Centrifugation at 10^3g for 30min 2) Recovery of the supernatant	1) Lyophilisation (-60°C for 72h) 2) Recovery of the dry matter content	Preservation in glass vials protected from light and at room temperature	Dissolution in ultra pure water and sterilization by filtration (0,22µm)
Frontbox					

Non aqeous form: cold aqeous extraction

Sample	Preservation before extraction	Extraction	Lyophilisation and recovery of dry matter content	Preservation after lyophilisation	Before testing
Grinder	4°C	1) Small squares 2x1cm 2) Addition of water (c°=0.2mg/mL) 3) Orbital shaking for 24h at 23°C 4) Recovery of water extracts	1) Lyophilisation (-60°C for 72h) 2) Recovery of the dry matter content under vacuum	Preservation in glass vials protected from light and at room temperature	Dissolution in ultra pure water and sterilization by filtration (0,22µm)
Starting product	Room temperature in aluminium foil				
End product					

Cytotoxicity

H^3-uridine uptake kinetics (NF EN 16418)

▪ **Reproductible and sensitive bioassay.**

Principle: Measuring the rate of incorporation of H^3-uridine over time (30min) during the RNA synthesis in cells. Positive substances are able to decrease RNA synthesis kinetic (decrease >30%).

➢ **Positive control** : potassium dichromate (2.5mg/L)

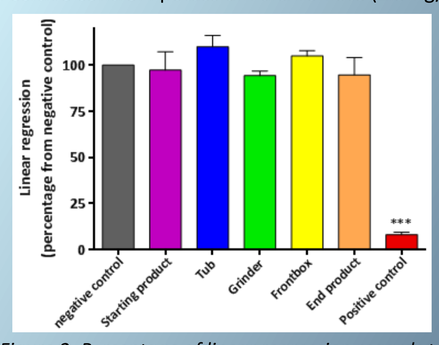


*Figure 2: Percentage of linear regression regards to negative control (100%) of HepG2 cells after 20h exposure to each extract of the production chain (concentration: 2mg/mL); five independent experiments; results +/- SEM; one sample t test to compare with theorical value of 100%, ***p<0.001*

No significative decrease of the HepG2 cells viability after exposure to samples of the production chain.

Genotoxicity

COMET ASSAY

▪ **Sensitive bioassay to detect and quantify DNA primary damage.**

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

➢ **Positive control :** metyl methanesulfonate $C_2H_6O_3S$ (50 µM)

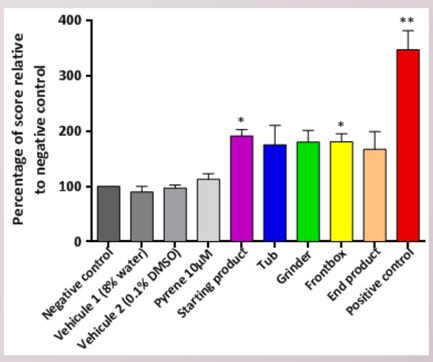
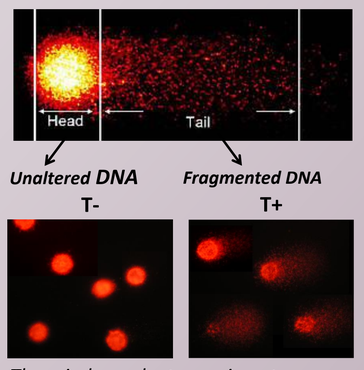


*Figure 3: Genotoxicity on HepG2 cells after 20h exposure to each extract of the production chain (2mg/mL). Three independent experiments; results +/- SEM; one sample t test to compare with theorical value of 100%, *p<0.05; ** p<0.01*



Three independent experiments were performed . The results are average and are expressed as percentage of a score relative to negative control (100%). 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).

All samples tend to increase the DNA damage of the HepG2 cells but the increases (2 fold) were only significant compared to the negative control with the starting product and the frontbox.

Oxidative stress

Dihydroethidium (DHE) and DCFDA labelling assays

▪ **Quantitative bioassays to detect oxidative stress thanks to the labelling with the dihydroethidium (DHE) and the DCFDA probes.**

Principle: Detecting cellular reactive oxygen species after labelling with the DHE probe and analyse by flow cytometry, or the DCFDA probe and analyse by fluorescence.

➢ **Positive control:** tert butylhydroperoxide (tBHP 15 µM)

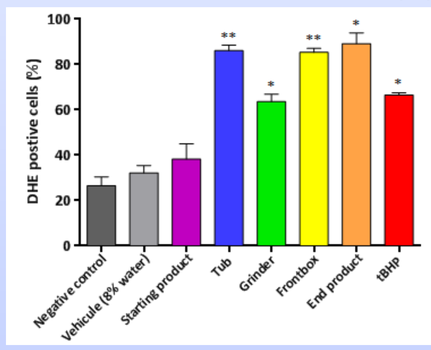


*Figure 4: Percentage of DHE positive HepG2 cells after 20h exposure to the extracts of the production chain (2mg/mL). 10 000 events counted by flow cytometry). Five independent experiments; results +/- SEM; Mann-Witney test to compare samples with negative control, *p<0.05; ** p<0.01; ***p<0.001*

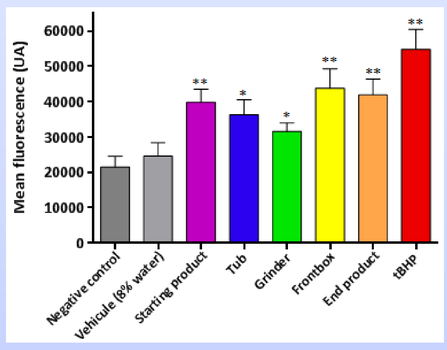


*Figure 5: Mean fluorescence after labelling with DCFDA and 20h treatment of HepG2 cells with the extracts of the production chain (2mg/mL). Eight independent experiments; results +/- SEM; Mann-Witney test to compare samples with negative control *p<0.05; ** p<0.01.*

DHE assay: significant increase of the percentage of DHE positive cells induced by treatment with the tub (x4), grinder (x3), frontbox (x4) and end product (x4), except the starting product, compared to the negative control. Nevertheless, when taking into account the mean fluorescence (data not shown), the treatment with the starting product significantly increased this parameter in comparison with the negative control.

DCFDA assay: significant increase of fluorescence after treatment with each extract of the chain.

Endocrine disruption

▪ **Highly sensitive and reliable bioassays.**

Stably transfected human estrogen receptor-alpha transactivation assay (OCDE 455)

Principle : Use of a stably transfected human cells with estrogen receptor-alpha (hERα) HeLa 9903 cells to detect estrogenic agonist or antagonist - activities of substances.

➢ **Positive control** : 17β-estradiol (E2 1nM)

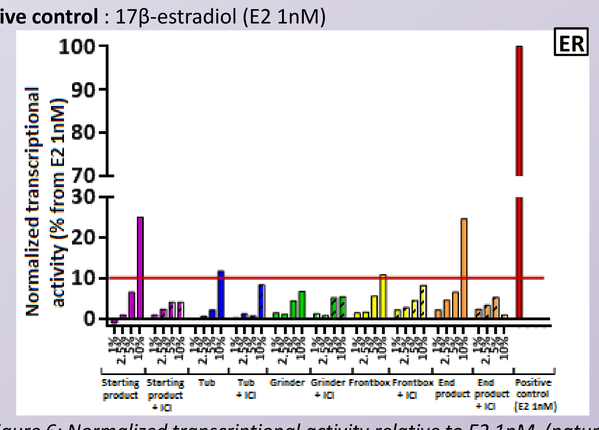


Figure 6: Normalized transcriptional activity relative to E2 1nM (natural reference agonist 17ß-estradiol) after 20h exposure with different concentrations of the extracts of the production chain (tested doses: 1, 2.5, 5 and 10% in the treatment media; highest dose 10%: 2.5mg/mL) in the presence or not of the specific inhibitor of hERα (ICI 182,780: fulvestrant). One experiment; positive effect: transcriptional activity > 10% (red line).

Without ICI 182,780: transcriptional activity superior than 10% after treament with the highest dose of four extracts: the starting product (25%), the tub (12%), the frontbox (11%) and the end product (25%). With ICI 182,780: transcriptional activities were strongly inhibited but only with the starting product and the end product in the presence of a specific inhibitor of hERα.

No extracts had antagonist activities (data not shown)

Stably transfected human androgen receptor transactivation assay (Wilson *et al.*, 2002)

Principle : Use of stably transfected human cells (MDA-kb2 cells) with the androgen receptor (hAR) in order to detect agonist or antagonist transcriptional activities on the hAR with substances .

➢ **Positive control** : hydroxyflutamide (HF 50nM)

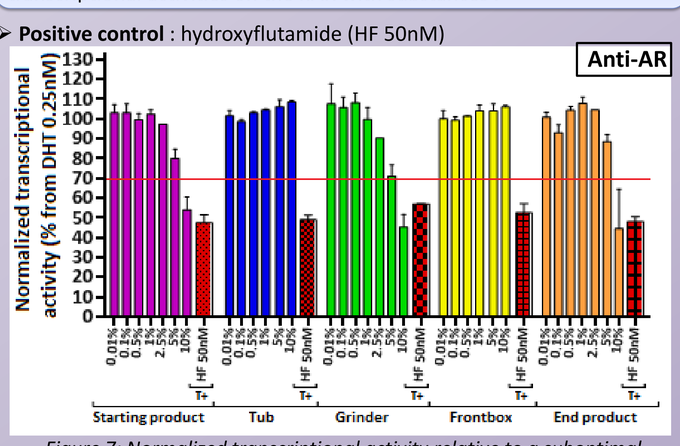


Figure 7: Normalized transcriptional activity relative to a suboptimal concentration of DHT 0.25nM (natural reference agonist 5α-dihydrotestostérone) after 20h exposure with different concentrations of all extracts of the production chain (tested doses: 0.01, 0.1, 0.5, 1, 2.5, 5 and 10% in the treatment media; highest dose 10%: 2.5mg/mL). Two experiments; positive effect: transcriptional activity < 70% (red line).

Agonist activities of the extracts have been evaluated and none of the samples were agonist of the hAR (data not shown). Tested samples had anti-androgenic activities as most of endocrine dirsuptors (figure 7).Transcriptional activity lower than 70% after treatment with the highest dose of three extracts : the starting product (50%), the grinder (45%) and the end product (40%).

Discussion/Conclusion

Cytotoxicity

None of the samples were cytotoxic for the HepG2 cells.

Genotoxicity

Comet assay: significant increase of primary DNA damages with two samples : the starting product and the frontbox.

Oxidative stress

DHE and DCFDA labelling assays: oxidative effects of all the extracts of the production chain.

⇨ Genotoxic effects of tested extracts observed with Comet assay may be due to oxidative stress induced by treatment with these extracts.

Endocrine disruption activities

- Estrogenic agonist activity of the starting product and the end product.
- Androgenic antagonist effects of the starting product, the grinder and the product of the end of the production chain.
- Estrogenic antagonist and androgenic agonist activities of the extracts have been evaluated but none of the samples gave positive effects with these assays.

Perspectives

- Ongoing chemical analysis of the extracts to identify which substances are responsible of the effects observed with the bioassays.
- Study the dose-effect relationship of the extracts which gave positve effects in bioassays.