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Een snelle *in vitro* biologische test voor controle van dioxine-achtige stoffen in voeding.
(An in vitro bioassay for screening dioxin-like substances in food samples)

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Executive Summary

Healthy products and food safety is what consumers expect from agriculture and the food industry. Adequate controls to protect public health, based on scientific knowledge, are a major task for the authorities.

This report describes the results of a three years study, performed at Vito (the Flemish Institute of Technological Research), which aimed to develop a monitoring tool to detect elevated concentrations of persistent polyhalogenated aromatic hydrocarbons (PHAHs) with dioxin-like activity in food products. This project was initiated well before the dioxin crisis. The Belgian dioxin crisis was a sinister demonstration of the urgent need for adequate food monotoring programs for dioxins and related compounds in human food products. The benefits of an adequate control program for society, for economy and for public health has been widely accepted now. It fits into the framework of sustainable development.

As a result of this study we have now available a validated bioassay which is ready for use to screen different food items for their concentration of compounds with dioxin-like activity. After comparing various bioassays we chose for the CALUX assay, based on genetically engineered rat liver cells cultured *in vitro*. The cells emit a light signal that can be quantified if dioxin-like substances bind to the intracellular arylhydrocarbon (Ah) receptor. The binding affinity to the receptor has been shown directly related with toxic potency. The most toxic dioxin congener (tetrachloro-p-dioxin = 2,3,7,8 TCDD) has the highest known binding affinity and is used as a standard in each series of measurements. The results of the bioassay measurements are expressed in toxic equivalents of 2,3,7,8-TCDD (TEQ). The cellular method was optimised and miniaturised to allow routine-analyses in 96- well plates. A further step was to expose the cells to PHAHs, which are present in milkproducts, in eggs, and meat. A standard procedure was developed for fat extraction and further isolation of the compounds from the fat extract. This step was necessary to overcome possible interference of the light signal with compounds of the food matrix. A major challenge was to obtain low background signals and a low limit of detection, this was technically more difficult than initially expected. Starting from 1 g of animal fat, we obtained a limit of detection of about 0,1 pg TEQ/ g lipid and a limit of quantification of about 0,4 pg TEQ/ g lipid which is comparable to the results obtained with chemical analysis of dioxins/ furans with HRGC-MS. The repeatability (variability of samples analysed in the same run) showed a coefficient of variation (CV) of 10%, intralaboratory reproducibility based on independent runs of the same samples showed more variation (CV of 26% for samples above 2 pgTEQ/ g lipid).

Results from the bioassay have been compared to results from chemical analyses (HRGC-MS) on the same food substances (62 milksamples, 17 cheese samples, 6 samples of animal fat, 5 samples of eggs). In milk samples, a significant correlation ($p<0,0001$) was found between CALUX-TEQ and TEQ-dioxins/furans, TEQ- PCBs and TEQ (PCDD/F + PCBs) with respective Spearman ‘s Rank correlation coefficients of 0,72, 0,67, 0,73. Results from eggs were also significantly correlated. No significant correlation was observed with the cheese data and the meat fat data. This may be due to the low concentrations measured in the cheese samples (average below 1 pg TEQ/ g lipid) and the few meat samples that were analysed within a narrow concentration range. All samples which showed chemical TEQ values above the current limit values in Belgium showed elevated CALUX TEQ concentrations, above 6 pg TEQ adjusted on a lipid weight basis. No false negative results were obtained.

The CALUX- TEQ values were higher than the chemically determined PCDD/F-TEQ or the PCB-TEQ. However if the TEQ values of PCDD/F and PCBs were combined, the CALUX TEQ was somewhat lower. The bioassay measures directly the dioxin activity of the mixture of compounds present in the extracts, taking into account dioxins, furans and coplanar PCBs and their possible interactions. This may be toxicologically more relevant than the information obtained from chemical measurements. It certainly guarantees a more conservative approach than if only dioxins/furans TEQ values are determined as is usually done.

Recommendations

1. *Based on the good correlation between CALUX -TEQ and chemically measured TEQ levels the CALUX bioassay can be recommended as a screening tool for routine measurement of potentially toxic PHAHs in food The bioassay allows to screen rapidly a large number of food samples. Positive samples are identified. The bioassay is however not specific. In order to identify the compounds that contribute to an elevated CALUX signal, subsequent chemical analyses are needed to identify the nature of the compounds that contribute to the toxic signal. We suggest a protocol in which chemical analyses are limited to those food items that turn out positive in the bioassay. These costly and laborious chemical analyses should be applied on positive samples, which are definitely the relevant ones for further research. Since no false negatives are identified with the bioassay in our study, this approach can be highly recommended as a result of our study.*

2. *If CALUX TEQ results are increased in comparison with the results from chemical analyses (marker PCBs or PCDD/F- TEQ), the samples may need further follow up to identify the chemicals which contribute to the CALUX signal. It may be possible to identify contamination with other persistent halogenated hydrocarbons that are not included in the conventional package of chemical measurements.*

In a second part of the study, various meat samples(34 samples) and fishery products (34 samples) were purchased from different food stores and processed for CALUX measurements. The study was too limited to obtain representative data for the selected food items and to draw definite conclusions. Meat samples contained average levels of CALUX- TEQ between 1 and 2 pg TEQ/g lipid, fish samples showed concentrations that went up to 39 pg TEQ/g lipid. If expressed per g fresh tissue, median concentrations were highest in herring (2,2 pg TEQ), followed by pork (0,7 pg TEQ) and eggs (0,7 pg TEQ). For risk assessment (contribution to TEQ body burden) we need to take also into account the relative contribution of these food items to the average diet. Using published food consumption data from abroad (since no up to date food consumption data exist in Belgium), combined with the median TEQ concentrations measured in this study, daily TEQ intake levels were calculated. Due to the above mentioned limitations they should be considered only as indicative.

Apart from the absolute TEQ values, interesting information can be obtained from the variability among samples from a selected foodproduct.

Pooled samples (milk, eggs) showed relative low coefficients of variation, while CV of individual milk and chicken samples went up to 0,80 and 0,50 respectively. Beef and pork

samples showed less variation. Fish samples also, had high variation coefficients, except for the salmon samples.

The bioassay allows to chararcterise a group of food items based on their TEQ concentration and on the heterogeneity among samples in TEQ values. This information allows prioritisation of food items that need further follow up.

Recommendation

3. *This assay can be used to detect rapidly, unknown contamination in food samples and may help to identify yet unknown sources of contamination. We recommend to use this screening assay regularly on a representative set of samples. Samples should be analysed on an individual basis. Samples with high coefficients of variation indicate that part of the consumers may be exposed to elevated TEQ concentrations in their food which do not occur in food items from a different origin. Food items showing high coefficients of variation require further systematic screening in relation to their origin or their processing.*

4. *CALUX is a promising tool for monitoring as part of a food surveillance campaign or for study of environmental levels of PHAHs in animal fat tissue. The next step for further implementation is an international validation study which is a prerequisite for further acceptance.*