Final Result of the European Research Project DIFFERENCE: New Alternative Methods for Dioxin Analysis

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Introduction

The European research project DIFFERENCE ("Dioxins in Food and Feed – Reference methods and New Certified Reference Materials") was focussed on the development of alternative methods for the analysis of polychlorinated dibenzodioxins (PCDDs), dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs) using comprehensive multi-dimensional gas chromatography (GC´GC), gas chromatography combined with low resolution ion-trap mass spectrometry (GC-LRMS/MS), the CALUX bioassay and an Ah-PCR technique. GC combined with high resolution mass spectrometry (HRMS) was used as a reference method in all comparisons. Given the need for a regular monitoring of PCDD/Fs and PCBs in Europe, which was further enhanced by the implementation of the maximum residue levels (MRLs) for PCDD/Fs in food and animal feed per 1 July 2002[1,2], and the relatively high costs of GC-HRMS and the limited capacity for HRMS analyses in European laboratories, cheaper, faster, but reliable methods were badly needed. The method development part included a developmental phase, a validation phase and a standardisation phase. In addition to the method development, attention was also paid to alternative extraction and clean-up methods. Finally, the DIFFERENCE project also included a feasibility study on the preparation and certification of five reference materials. This part was included because at the moment no certified reference materials (CRMs) are available that cover all PCBs, PCDDs and PCDFs included in the European legislation.

More specifically, the above mentioned objectives could be subdivided in:

1. Identification and selection of food and feed matrices in view of dioxin/PCB contamination and description of these matrices for the purpose of:
2. Assessment of the feasibility of the preparation of these materials as CRMs by the preparation of test batches, including homogeneity and stability studies
3. Optimisation of bio-analytical and chemical screening methods for dioxin and PCB analysis. The methods included were:

a) GCxGC-ECD (or ToF-MS)

b) GC-LR MS(/MS)

c) CALUX bioassay

d) Ah-PCR assay

4. Assessment of the measurement capability of laboratories to certify the candidate CRMs
5. Validation and standardisation of the developed and optimised screening methods, including the extraction and clean-up methods
6. Development, optimisation and validation of extraction and clean-up methods for dioxin and PCB analysis and combination of these methods with the developed screening methods or with a GC-HRMS method. The methods included were:

a) Accelerated Solvent Extraction (ASE)

b) Microwave Assisted Extraction (MAE)

c) Supercritical Fluid Extraction (SFE)

Selection of test materials

The selected materials for the feasibility study on certification were: a fish tissue (herring), pork, milk, a fish oil and a compound feed. The first three materials were canned, the fish oil was ampouled and the compound feed was packed in plastic screw-cap jars. In addition, a large number of test materials for WP3 including a series of PCB and PCDD/F standard solutions, blanks, and solutions with possible interfering compounds were prepared.

Homogeneity and stability tests

The homogeneity and stability tests, based on the analysis of the target compounds, revealed no specific problems that could hinder the certification of reference materials for the PCBs and PCDD/Fs which are currently included in the European legislation for food and feed. All preparations went smoothly. Some homogeneity problems were identified for the canned milk. These can be solved by using a milk powder instead of canned milk as a candidate CRM.

Development and validation of alternative methods

The strategy of the DIFFERENCE project was to start from four different alternative techniques for the determination of dl-PCBs and PCDD/Fs, supposing that at least one of them would turn out to be suitable to serve as an alternative method for GC-HRMS. That technique should then be combined with the improved extraction and clean-up to speed up the analysis and to further reduce analysis costs in that way. The results of WP 3 have surpassed these expectations: three out of the four methods have successfully been developed and validated[3].

CALUX may be considered as a valuable screening method that can be used in times of crisis for a large number of samples to indicate if those samples are below, around of above the EU MRLs. Quantitative total-TEQ values may also be produced by this method, but due to the variability of those data, in particular for the mono-ortho substituted PCBs, the use of CALUX in that way is not recommended. The application of recovery correction appeared to be essential in any case[4].

GC-LRMS/MS may be used as a reliable routine method that will produce congener-specific data. Excellent results have been achieved in the various interlaboratory studies and the feasibility study on certification. However, a few critical observations have to be made. During the project basically one laboratory has worked on this technique. The results were very good, but confirmation by other laboratories is needed. It may well be that only the specific type of GC-MS/MS used produces good results whereas other types may not deliver such results. Furthermore, a highly experienced technician is needed to ensure good results and the maintenance of the MS and particularly of the ion-source may be time-consuming because cleaning of the source may be required regularly.

Finally, GCxGC-ECD or GCxGC-ToF-MS emerged as viable routine methods for dl-PCBs and PCDD/F measurement. No doubt for environmental samples, and for food and feed materials in which dioxins and dl-PCBs occur at higher concentrations (around the MRLs in fatty fish, fish oil),GCxGC is able to serve as an excellent routine method and alternative for HRMS[5]. As soon as dioxin and dl-PCB concentrations drop to low levels, i.e. lower than the MRLs and action levels, e.g. in lean fish, compound feeds, and meat, a lot of time is needed to produce congener-specific data, to integrate the chromatograms, to adjust the baseline, to compare relative retention times, etc. However, even then GCxGC may still serve as a screening method. Better software will help to speed up the data acquisition at lower concentrations, but some identification problems may remain in the near future for relatively clean materials. GCxGC-ToF-MS is not so much more sensitive than GCxGC-ECD. However, the peak shapes are better because the peaks do not suffer from the relatively large cell volume of the micro-ECD that causes peak tailing. Also, the additional information from the mass spectra helps to identify the target compounds at low concentrations. In that sense, GCxGC-ToF-MS is an improvement compared to GCxGC-ECD, but obviously the instrument is considerably more expensive. The work on GCxGC has created a lot of off-spin from this project. In addition to the application for dioxin analysis, this technique can be used in many applications in which complex mixtures need to be analysed. Excellent chromatograms have been achieved for other contaminant mixtures such as toxaphene, chlorinated paraffins, polychlorinated naphthalenes, brominated flame retardants and many others. Also, these contaminants can be separated from each other, enabling a multi-component analysis. In that way GCxGC is invaluable. It is expected that in the near future many environmental and food-safety laboratories in Europe and

world-wide will implement this technique in their methods. Other applications of GCxGC have been seen in the oil industry and, e.g., in the separation of fatty acids.

The fourth method that was studied, an Ah-PCR analysis, is still under development. It is not excluded that that technique may finally also result in a suitable alternative screening method. The research on this technique was delayed by several managerial problems.

Feasibility study on the preparation and certification of materials for PCBs and PCDD/Fs

The certification feasibility study has resulted in good perspectives for a final certification. This part of the project also showed that it was possible to use the alternative techniques GC-LRMS/MS and GCxGC-ECD for certification, in addition to GC-HRMS.

Alternative extraction and clean-up methods

The WP on alternative extraction and clean-up methods has focussed on the development of pressurized liquid extraction (PLE), also called accelerated solvent extraction (ASE). It appeared that this technique is to be preferred to supercritical fluid extraction (SFE) and microwave assisted extraction (MAE). In particular the addition of a fat retainer to the extraction cell is a major step forward because in that way one or more clean-up steps have become redundant and valuable technician time is gained[6]. A further improvement is the inclusion of a carbon column into the extraction cell, through which, after back-flush, the target compounds (planar PCBs and PCDD/Fs) are eluted[7]. In that way it is even possible to do the extraction within ca. 10-15 minutes, concentrate the extract and, without any further clean-up go to the GC/MS, GCxGC or CALUX assay for analysis of the extract. Per series of 10-15 samples, this saves ca. 3 days work of a technician. The gain in time achieved in this way is considered as the most successful result of this project.

Standardisation

In order to start the standardisation process, contacts have been made with the CEN, technical working group 275. CEN has responded positively on the proposal to standardize the developed methods, as well as the reference method (GC-HRMS). However, due to the substantial variation in the results of the CALUX assay, it may be difficult to standardise this assay within CEN. Further work on the standardisation will be carried out in the near future.

Apart from a judgement on the scientific quality of the results of the alternative methods, DIFFERENCE has also addressed the economical aspects of the methods. An evaluation of costs of investment, manpower, analysis time etc. has been made for all methods developed including the new extraction and clean-up techniques. The most important observations are: i) The major gain in time and costs was achieved by the application of the ASE technique in combination with a fat retainer, and, possibly, in combination with a small carbon cell. This approach saves 2-3 days of a technician per series of 10-15 samples. It can be used in combination with GC-HRMS, GCxGC-ECD or –ToF-MS, GC-LRMS/MS, and CALUX; ii) In times of crises with many samples to be screened for the presence of dioxins and/or dl-PCBs CALUX is a rapid and useful method which cannot be beaten at the moment by any other method. However, it should not be used for quantitative purposes, but just to detect dioxins and/or dl-PCBs around or above the MRLs or to confirm the absence of these contaminants; iii) GC-LRMS/MS is much cheaper than GC-HRMS in investment in instrumentation and maintenance costs; there is not so much reduction of man hours compared to GC-HRMS; iv) GCxGC-ECD is considerably cheaper in investment in instrumentation than GC-HRMS. However, for low level samples the costs for the interpretation of the chromatograms are, for the time being, considerably higher than that in GC-HRMS. Better software may help to reduce the amount of time needed. For environmental samples, and for more fatty food and feed samples with concentrations at levels around the MRLs or higher, this method is an excellent alternative to GC-HRMS. GCxGC-ToF-MS is a proper alternative that delivers more structural information. However, the instrument costs are relatively high, although not as high as that of a GC-HRMS.

Conclusions

The DIFFERENCE project was born at a time in which GC-HRMS was the only available technique for dioxin analysis. The CALUX assay was available as a non-validated screening technique. GCxGC was very much in its infancy and LRMS seemed not to be suitable due to its low sensitivity, in particular for 2,3,7,8-TCDD. Extraction and

clean-up were all done in the traditional way. Now, almost five years later, there is ASE, a much faster, reliable extraction and clean-up method, there is a validated CALUX screening method, and there are two methods that can be used as a routine method for dioxin and dl-PCB analysis: GCxGC and GC-LRMS. There is a good basis for the certification of reference materials for all dioxins, furans, and dl-PCBs included in the European MRLs for food and feed in five different materials. Finally, a standardisation process has started for the two routine methods as well as for GC-HRMS.

Nevertheless, a number of critical remarks should be made as well. GCxGC-ECD is a fine method, but is still time-consuming when used for samples with low levels of dioxins and dl-PCBs. Further work on software development and on improvement of the sensitivity will be needed. GCxGC-ToF-MS is a proper alternative, but expensive. GC-LRMS/MS has produced very good results in this project, but basically only one laboratory has worked on this method. It should be studied if the method is robust enough when used in other laboratories and if other types of instruments would meet the same standards. For several reasons time was too short to develop the Ah-PCR technique sufficiently. More time is needed to study and improve this alternative, which is promising, provided the effect of interferences can be reduced. Over the last five years other assays or techniques for contaminant or toxin analysis have been developed. Some of these seem to have enough potential to serve as an alternative rapid method for dioxin analysis. It may be worthwhile to test a selection of those new techniques in the near future.

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