IS STANDARDIZATION OF EXTRACTION METHODS FOR DETERMINATION OF PCDD/FS AND PCBS IN FOOD REQUIRED?

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Introduction

The current EU regulations and recommendation set maximum and action levels for the concentrations of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs), dioxin-like polychlorinated biphenyls (PCBs) and indicator PCBs on fat and wet weight basis[1,2]. Maximum and action levels for food of animal origin, except for muscle meat of fish and fishery products, fish liver and liver of certain terrestrial animals, are set on fat basis. For the above mentioned excluded food products, for foods for infants and young children, clays as food supplement, fresh fruits, vegetables and herbs, and cereals maximum and action levels are set on wet weight basis. Action levels expressed on product as sold are defined for dried fruits, vegetables and herbs.

For food with legal limits based on fresh weight or products thereof a complete extraction of the analytes of interest from the representative sample aliquot is obligatory while a complete fat extraction, if applicable, is not essential. If maximum and action levels are based on fat, additionally the fat extraction has to be considered, as this can have significant influence on the results for the analytes of interest.

EU regulations follow a criteria approach for determination of PCDD/Fs and PCBs in feed and food and therefore specific extraction methods, especially also for lipid extraction from food samples, are not defined. Established analytical criteria require only an appropriate validation of the extraction efficiency, depending on the stage at which internal standards are introduced and whether results are reported on product or fat basis.

Similar analytical criteria are also defined for animal feed. Additionally, for these matrices, extraction methods are described in EN 16215:2012[3].

Results of proficiency tests organized by the European Union Reference Laboratory (EU-RL) for Dioxins and PCBs in Feed and Food, and further extraction tests carried out at EU-RL have shown that for certain matrices, the applied extraction method can have a considerable influence both on the extracted lipid content and on the results for the analytes of interest. Specific provisions ensure consistent results and can either be based on a detailed step-by-step description of the applied method or on the definition of specific criteria for the extraction of analytes of interest and fat.

Materials and methods

Extraction tests for several representative food matrices of animal origin (liver, meat, eggs and milk) were conducted at the EU-RL for Dioxins and PCBs in Feed and Food applying different methods for sample pre-treatment and extraction, and several different extract solvents and solvent mixtures. Sample pre-treatment included freeze-drying and mixing with drying agent; Twisselmann hot extraction, pressurized liquid extraction, Ultra Turrax extraction and liquid/liquid extraction were applied. As extraction solvents a wide range of non-polar and polar extraction solvents and solvent mixtures were tested. A detailed description can be found elsewhere[4].

Between 2009 and 2013 the EU-RL organized five proficiency tests on the determination of PCDD/Fs and PCBs in food matrices of animal origin. The following food matrices were tested:

- pork sausage
- salmon filet
- whole egg
- egg yolk powder
- milk powder

Between 77 and 124 laboratories participated in these proficiency tests using GC-HRMS confirmatory methods, GC-MS and bioanalytical screening methods for detection of the analytes of interest, and reporting at least one of the requested parameters. Evaluation of results included the analytes of interest (PCDD/Fs, DL-PCBs,

indicator-PCBs) and the lipid content. Various pre-treatment and extraction methods as well as extraction solvents/solvent mixtures were applied by the participants.

Results and discussion

Extraction tests at the EU-RL:

For lamb liver with a high proportion of phospholipids in the total lipid content, the EU-RL could demonstrate that different suitable solvents/solvent mixtures extract the analytes of interest to a comparable degree, but they extract different kinds of lipids in different quantities as well. As a consequence, the concentrations of dioxins and PCBs on fat basis in sheep liver were clearly dependant on the extraction method and/or solvents applied and therefore on the amount of extracted fat[4]. When comparing results on wet weight basis, however, the levels of dioxins and PCBs were quite comparable. Based on these observations, in Commission Regulation (EU) No 1067/2013 of 30 October 2013 maximum levels for liver of terrestrial animals are set on wet weight basis for the first time. This regulation also refers to the above mentioned findings of the EU-RL[1].

For milk and hen's eggs variations of the extracted lipid content were observed, but those were considerably lower as for lamb's liver. For hen's eggs with a proportion of 25 % of phospholipids in the total lipid content slightly higher lipid fractions were found when extracted with polar/non-polar solvent mixtures. For homogenized milk the presence of small fat globules can have an additional influence depending on the applied extraction solvent. No significant effects of the application of different extraction solvents could be shown for beef[3].

Results of proficiency tests:

The statistical evaluation of the PT results is performed according to ISO 13528[5] and the IUPAC protocol[6] as described elsewhere[7]. For checking of the performance of participating laboratories regarding the analyte/lipid extraction and determination of the lipid content, the reported results for PCDD/Fs and PCBs are compared with the reported lipid content taking into consideration the applied extraction methods and solvent properties.

The above mentioned matrices of food of animal origin covered a range of lipid contents between 6 % (salmon filet) and 56 % (egg yolk powder). For pork sausage, salmon filet, whole egg and egg yolk powder the relative robust standard deviation (outliers removed) for the lipid content was about 10 %. Based on all reported results the CV ranged between 12 % (egg yolk powder) and 34 % (salmon filet). For milk powder an assigned value for the lipid content could not be calculated due to the relatively wide distribution of participants' results.

The distribution of participants' results for WHO-PCDD/F-PCB-TEQ and the lipid content for these food matrices is shown in figure 1.

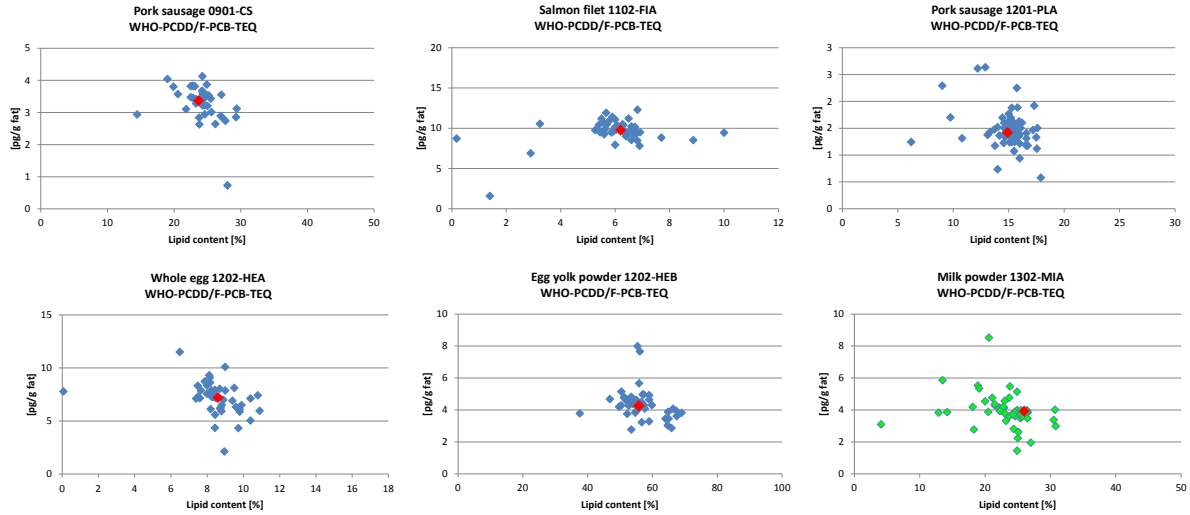

Figure 1: Correlation of participants' results for WHO-PCDD/F-PCB-TEQ [pg/g fat] and lipid content and comparison with assigned values (red), for milk powder comparison with fat content obtained from reference method (red)

The evaluation performed for salmon filet showed, that results for analytes within acceptable ranges on fat basis do not automatically lead to correct results on fresh weight basis. For the food matrices with results expressed on fresh weight basis a complete extraction of the analytes of interest from the matrix is obligatory. When results have to be expressed on fat basis, also a partial extraction of analytes of interest and lipids in the same way can lead to correct results on fat basis. However this cannot be concluded automatically for all concerned matrices and therefore complete extraction of analytes of interest and fat is recommended.

Further investigation of the results for milk powder showed, that the calculated robust mean and median of participants results for the lipid content were considerably below the declared value of 26 % or the value obtained from the reference method for determination of milk fat (26.2 %). About 85 % of lipid contents reported by participants were below these values. As reference method for the determination of the fat content EN ISO 1736:2008 (Dried milk and dried milk products - Determination of fat content - Gravimetric method)[8] was applied. The method is based on the extraction of an ammoniacal ethanolic solution of milk powder with diethyl ether and petroleum ether and the gravimetric determination of the lipid content.

Participants applied a wide range of extraction methods, including pre-treatment of the milk powder before extraction, extraction techniques and extraction solvents or solvents mixtures. Acceptable results for the lipid content were obtained for various combinations of pre-treatment technique and solvents.
When correlating extraction solvents/solvent mixtures with the obtained lipid contents and concentrations for analytes of interest (see figure 2), it could be shown that results for comparable extraction solvents can result in different lipid contents (and TEQ concentrations). Results seem to be highly depending on the pre-treatment and extraction method.

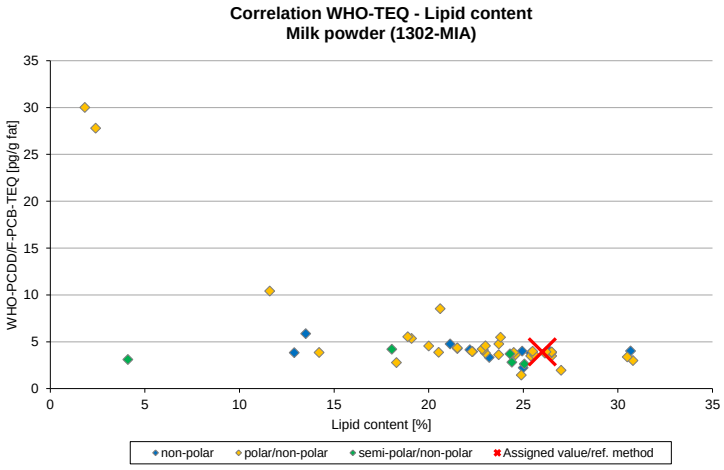

Figure 2: Correlation of WHO-PCDD/F-PCB-TEQ [pg/g fat] with the lipid content [%] for milk powder in comparison with polarity of applied extraction solvents/solvent mixtures.

Standardization of extraction methods

Currently EU regulations don't set specific criteria for extraction methods or determination of the lipid content or define certain methods for extraction in detail. They only refer to an appropriate validation of the extraction efficiency. For example, in EPA Method 1613, Revision B[9] for fish and other tissue two different extraction methods are defined, which are based on Soxhlet extraction or shake extraction after HCl-digestion with methylene chloride/hexane as extraction solvent.
As can be seen from the results of proficiency tests and extraction tests at the EU-RL, a standardization of extraction methods or the definition of specific criteria in the framework of the criteria approach as defined for the analytes of interest, is necessary for the comparable extraction of specific matrices.

Most important criterion for the application of extraction methods is the complete extraction of the analytes of interest, either on fat or on fresh weight basis. This can be achieved by a criteria approach with definition of certain requirements for extraction efficiency or the detailed definition of extraction methods (or a combination of both approaches).
The following issues and questions come up in connection with the standardization of extraction methods:

- Is a complete lipid extraction and determination of the total lipid content always necessary or is the extraction of an aliquot of analytes and lipids in some cases sufficient?
- How can the completeness of extraction for analytes and fat be checked? Proficiency tests and/or comparison with established reference methods?
- What is the correct fat content and how can it be determined? Using a reference method or the method applied for PCDD/F and PCB analysis?
- Can the fat extraction be used as criterion for the correct extraction of the analytes? What criteria can be applied for checking of correct fat extraction?
- How to deal with the determination of the fat content and extraction of analytes with separate extraction methods?

In case of standardization of the extraction methods for the relevant (regulated) matrices, it is possible to define only general principles of the extraction method, e.g. extraction techniques, extraction solvents, pre-treatment, in combination with specific criteria, or the complete method in detail. Focus should then be on the exact definition of critical steps, as proficiency tests showed that the same extraction methods can lead to considerably different results. For some of the relevant matrices also standardized methods are established for the determination of the fat content. Suitability of these methods should be checked for unchanged use or application after appropriate adjustments for extraction of PCDD/Fs and PCBs.

Conclusions:
Results of extraction tests with different extraction techniques and solvents as well as results of proficiency tests showed, that the extraction efficiency of methods can differ considerably. This can have an effect on the extraction of fat, but - even more important - also on the results for the analytes of interest.
Therefore the network of EU-RL and NRLs initiated upon request by the European Commission discussions on specific provisions for the extraction of fat and the analytes of interest.

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