SUMMARY OF LA LETTRE DE CECALAIT, N° 35 (4th quarter 2000)

(Translation : A. BAPTISTE, Correction : H. LAMPRELL)

The dioxin problem

(Abstract of the lecture given by Mr Fraisse at CECALAIT's Annual General Meeting 2000)

Structure and chemical properties

Dioxins, or polychlorinated dibenzo-*p*-dioxins or PCDD are a family of molecules based on the structure given in fig. 1, p. 1 in La Lettre de CECALAIT. The two benzene molecules may be substituted by Cl atoms at 8 different places, thus building a family of 75 congeners. The word « dioxin » also refers to another family : the furans or polychlorinated dibenzofurans or PCDF, with a relatively similar structure (cf fig. 2, p. 1 in La Lettre de CECALAIT). These molecules may also be substituted, building a family of 135 congeners.

Among these 210 (75 + 135) different molecules, some are 2-3-7-8 substituted. Their very planar and stiff structure gives them an extreme resistance towards chemical and biochemical processes. Consequently they persist in the environment and bioaccumulate in the food chain. 10 furans and 7 dioxins, called the dirty dioxins, have that conformation, in particular the most toxic and studied congener, 2,3,7,8 TCDD – *Seveso dioxin*-.

These molecules are not very soluble in water (the more Cl atoms, the lower solubility), not very volatile and have a high chemical and thermic stability. They are lipophilic, soluble in organic solvents and degraded by UV.

Origin

Dioxins are formed during combustion processes of organic products in the presence of chlorine : volcanic eruptions, forest fires and mostly domestic waste incineration. Moreover, they are unwanted byproducts of industrial processes : preparation of herbicides and bactericides, bleaching of paper pulp. Finally, they may also be produced accidentally in chlorophenol preparation (Seveso accident) or when strongly heating polychlorinated biphenyls (PCB) widely used industrially because of their dielectric properties.

They are found in the air, water and earth. In the air, their half-life is a few days, but after deposition, they may contaminate waters and earth, where their half-life is about 1 to 3 years on the surface and more than 10 years when below. Accumulation in plants was never observed, but it is the case in seaweed. Animals and man are contaminated mainly through food and dioxins tend to be adsorbed in fatty tissues. The evaluation of human exposure considers either all possible environmental sources or, as exposure from food is the main pathway –90%-, dietary intakes.

Toxicity and human exposure

The toxicity of dioxins may come from several, still unknown ways. At the biomolecular level, there is, however, one well-known way : dioxins enter the cell and bind to an arylhydrocarbon receptor. This complex can then bind to a dioxin-responsive element on the DNA, which results in altered gene expression.

A broad spectrum of toxic and biochemical effects was observed in laboratory animals, especially for chronic exposure. TCDD is a carcinogenesis promoter, but sensitivity depends on species (for example, guinea pigs develop liver tumours whereas rats remain unaffected) and sex (for example, female rats develop liver tumours faster than males).

For human health, subtle biological and biochemical alterations may occur after low exposure, but their clinical significance is not yet known. Exposure to high levels, observed after industrial accidents (Seveso explosion) or accidental food contamination (Yusho rice oil) result in cloracne, neurological, developmental reproductive effects and increased cancer risks.

The general population is currently exposed to levels of dioxins several magnitudes lower than accidental levels, but exposure is always linked to complex mixtures of different dioxin congeners. So the concepts of TEQ (toxic equivalency quantity) and TEF (toxic equivalency factors) have been developed to facilitate risk assesment. The value of the TCDD TEF, the most potent and well studied dioxin congener, is 1. Other TEF are evaluated on the basis of toxicological database. TEQ of a given sample is calculated by the following expression :

$$ITEQ = \Sigma\ TEF_i \times C_i$$

where C_i is the concentration of congener i.

TEF of the 17 dirty dioxins range from : 1 for 2,3,7,8 TCDD and also for 1,2,3,7,8-PeCDD** (pentachlorodioxin), to 0.0001 for 1,2,3,4,6,7,8,9 OCDD (octachlorodioxin) or from 0.5 to 0.0001 for furans.

** *in the WHO 1997 list.*

TEF schemes are regularly revised. For example, EPA 1987 or NATO 1988 lists included only PCDDs and PCDFs and the TEF value of pentachlorodioxin was 0.5. However, since 1997, WHO and EPA recommend the inclusion of some dioxin-like PCBs (cf fig.3, p.3 in La Lettre de Cecalait). This is a group of 14 molecules (among the 209 PCB congeners) which have a coplanar conformation similar to PCDD or PCDF and also bind to arylhydrocarbon receptors. The TEF of PCBs are far lower than those of the PCDDs, but as their concentrations in the

environment are higher, they are likely to contribute significantly to the TEQ.

For the next revision (2003), WHO also recommends the inclusion of polybrominated dibenzodioxins.

Now, WHO recommends a tolerable daily intake of 1 to 4 pg/kg of body weight/day. This is calculated for a chronic exposure, ie a daily intake for a life for a 70 kg adult.

Determination of dioxins

It is well-known that it requires special expertise and sophisticated instrumentation. The most specific and sensitive method is gas chromatography coupled to high resolution mass spectrometry. For quantification, isotopic dilution with ^{13}C analogs is necessary.

Isotopic dilution is adding a known mass of a labeled compound to an unknown mass, to be determined, of the same native compound. This technique is recommended when high losses of the analyte may occur during extraction and purification steps. Indeed, both the native and the labeled compound will come through the same steps and have the same losses. Usually, samples are spiked with a mixture of the 17 ^{13}C labeled « dirty dioxins » immediately after grounding, sieving or homogeneization. But , for high-fat food, including dairy products, fat is spiked after extraction.

Fat contaminants are then separated and concentrated, either through different chromatography columns or through an activated charcoal column, which is longer, but enables to purify high fat quantities (about ten grams or more) and to detect very low dioxin concentrations.

Further cleaning up, especially for the removal of non « dioxin-like » PCBs requires other chromatography column steps. The final fraction should only contain PCDDs, PCDFs and some « dioxin-like » PCBs and is analysed by high resolution gas chromatography coupled to high resolution mass spectrometry.

Any accidental contamination of samples or reagents must be avoided. The fraction is spiked with another labeled standard, as a recovery standard prior to the gas chromatography, in order to check that the losses are under 50%.

Chromatographic profiles are obtained for native and labeled congeners, allowing the calculation of the initial concentration of each congener. Each is then multiplied by its corresponding TEF, and the sum leads to the TEQ of the tested compound.

There are other analytical methods, but which are not as sensitive. Screening and determining some PCBs, which is generally easier and faster than dioxin determination, as tracers of dioxin contamination is valid only if the source of the dioxin contamination is PCBs. Otherwise, no link between PCB and dioxin concentration could be shown.

The only current European standard is for dioxin determination in emissions. An ISO standard for determination in food is under preparation based upon the EPA 1613 method. Meanwhile, the method used for any dioxin determination must be carefully described.

Over the last few months, in several countries, there was a high concern with the estimation of dietary intakes of dioxins, resulting in several reports : one issued by the European Commission, 3 reports in France …. (see bibliography).

abbreviations

NATO : North Atlantic Treaty Organization
PCB : polychlorinated biphenyl
PCDD : polychlorinated dibenzo-*p*-dioxin
PCDF : polychlorinated dibenzofuran
WHO : World Health Organization

For other abbreviations and bibliography, please see page 4 in La Lettre de CECALAIT n° 35

AFNOR VALIDATION

AFNOR (French standardisation body) validated recently the following alternative methods :

↳ SPRINT ***Salmonella***, manufactured by Oxoid, validated on 2000/7/5 : a pre-enrichment and an enrichment kit for Salmonella, applicable to all dairy products.

↳ ALOA / L. Monodisk, manufactured by AES, validated on 2000/9/27, a chromogenic medium for the rapid detection of *Listeria monocytogenes*, applicable to food.

INTERESTING NEW STANDARDS

IDF STANDARDS

IDF 12C :2000 BUTTER Determination of salt content (Mohr method)

It is a joint IDF/ISO/AOAC, superseding 1988 provisional standard. The corresponding texts are ISO 1738 :1997 and AOAC 960.29.

IDF 141C :2000 WHOLE MILK Determination of milkfat, protein and lactose content. *Guidance on the operation of mid-infrared instruments*

It is a joint IDF/ISO/AOAC, superseding 1996 provisional standard. The corresponding texts are ISO 9622:1999 and AOAC 972.16.

IDF 156A:2000 MILK AND MILK PRODUCTS Determination of zinc content. *Flame atomic absorption spectrometric method*

It is a joint IDF/ISO/AOAC, superseding 1992 provisional standard. The corresponding ISO text is ISO/CD 11813.

IDF 185:2000 MILK AND MILK PRODUCTS Determination of nitrogen content. *Practical combustion method following Dumas principle*

This is a joint IDF/ISO/AOAC provisional standard (ISO/CD 14891)

IDF 182:1999 MILKFAT Preparation of methyl esters from fatty acids.

This is a joint IDF/ISO/AOAC provisional standard (ISO/CD 15884)

IDF 184:1999 MILKFAT Determination of the fatty acids composition by gas chromatography.

This is a joint IDF/ISO/AOAC provisional standard (ISO :DIS 15885).

IDF 183:1999 Guidance for the standardized evaluation of microbial inhibitor tests.

This is a joint IDF/ISO/AOAC provisional standard, but there are no corresponding ISO or AOAC texts at the time being.

ISO STANDARDS

ISO 12080-1 & -2 june 2000 DRIED SKIMMED MILK Determination of vitamin A content. Part 1 : Colorimetric method. Part 2 : Method using high-performance liquid chromatography

ISO 14378, june 2000 MILK AND DRIED MILK Determination of iodide content. Method using high-performance liquid chromatography

ISO 8196-1 & -2, june 2000 MILK Definition and evaluation of the overall accuracy of indirect methods of milk analysis. Part 1 : Analytical attributes of indirect methods. Part 2: Calibration and quality control in the dairy laboratory

ISO 3890-1 & -2, august 2000 MILK AND MILK PRODUCTS Determination of residues of organochlorine compounds (pesticides). Part 1 : General considerations and extraction methods. Part 2 : Test methods for crude extract purification and confirmation.

Results of the European Programme on staphylococci

(Abstract of the lecture given by Mrs De Buyser at CECALAIT's Annual General Meeting 2000)

Before accepting a standard as an European standard, CEN (*the EU standardization body*) requires precision data, which must have been validated by collaborative studies, as specified in standard ISO 5725. At the end of 1996, the European Community launched a 4 year project to validate six ISO microbiological methods for acceptance as standards. These are the methods of detection and/or enumeration of the following pathogens : *Bacillus cereus, Listeria monocytogenes*, coagulase positive *Staphylococcus, Clostridium perfringens*, *Salmonella.*

We have already reported on *Bacillus cereus* (see La Lettre de CECALAIT n° 26) and on *Listeria monocytogenes* (see La Lettre de CECALAIT n° 30). Now the studies on staphylococci are also finished.

Three contractors are involved in this project :

- AFSSA in France, also coordinator of the project,
- RIVM in the Netherlands,
- MAFF-CSL in the United Kingdom.

Among sub-contractors, CECALAIT was responsible for preparation, development, definition of preservation parameters and shipping of the cheese samples.

Coagulase positive staphylococci, ie mostly *Staphylococcus aureus*, but also some other species, are pathogens, able to produce enterotoxins, responsible for food poisoning. Dairy regulation, eg EC directive 92/46 specifies that they should not exceed « m » = 100/ml, in raw milk meant for human consumption when brought on to the market ; in other dairy products (when marketed) they should be less than 10, 100 or 1000/ml or /g depending on the type of product.

The horizontal reference method for their enumeration is ISO 6888, which has 2 parts. Part 1 uses Baird-Parker agar, then a confirmation test by a coagulase reaction. Part 2 uses the same base medium; supplemented with rabbit plasma and bovine fibrinogen solution (RPFA).

The studies were aimed at the determination of repeatability,r, and reproducibility, R, values for each of these methods.

↳ COLLABORATIVE STUDIES

As in the other studies of the project, the samples used were :

- reference material (capsules prepared by RIVM containing milk powder contaminated by *Staphylococcus aureus*),
- three different artificially contaminated food matrices :
  - raw milk cheese,
  - dried meat, prepared by MAFF-CSL,
  - dried food : whole egg powder, prepared by RIVM.

They were inoculated, at different inoculum levels, with one or several *S. aureus* strains, with coagulase negative staphylococci and also with a simulated autochthonous flora for cheese and meat.

The final contamination levels are given in table 1 in La Lettre de CECALAIT, page 8. Homogeneity and stability were checked before the beginning of the study.

The collaborative studies took place in june 1999 and involved 24 laboratories from 16 European countries. The analyses were made in blind duplicate and most laboratories performed both methods. Let us remind you of the principle of the methods :

- For ISO 6888-1 :
  - inoculation of the surface of two plates of selective solid Baird-Parker medium, with the initial suspension or serial dilutions,
  - aerobic incubation at 37°C and counting of typical and/or atypical colonies at 24 and 48h,
  - confirmation by coagulase test on some typical and/or atypical colonies.
- For ISO 6888-2 :
  - inoculation in the depth of two plates of selective RPFA agar, with the initial suspension or serial dilutions,
  - aerobic incubation at 37°C and counting of typical and/or atypical colonies at 24 and 48h,

↳ RESULTS

Shipping and reception of the samples were generally found satisfactory.

There were some discrepancies among laboratories concerning the differentiation between typical and atypical colonies on Baird-Parker medium.

After log transformation and exclusion of outliers, repeatability and reproducibility were determined. Calculation followed ISO 5725 as usual, but also standard project EN ISO 16140 (using the median value) which seems to fit better to microbiological methods.

The values obtained, using these two methods of calculation were almost the same. Tables 2 and 3 in La Lettre de CECALAIT, pages 8 and 9 show the results.

There are few differences between the different food matrices, except with cheese analysed with Baird-Parker medium. Repeatability and reproducibility are overall better with RPFA agar. Table 4, page 9 in La Lettre de CECALAIT shows the mean precision values obtained in the collaborative studies.

Let us remind you of the values obtained after grouping together the results of several CECALAIT proficiency studies (cf Lettre de CECALAIT, n°20) :

- r(log) = 0.52 and R (log) = 1.13 for Baird-Parker medium;
- r(log) = 0.19 and R(log) = 0.43 for RPFA medium.

They are higher than the values given above. But, we have to consider that :

- first, the participating laboratories were more diverse than those involved in the European project.
- then, a lot of participating laboratories hardly followed the standardized procedure, especially with Baird-Parker medium.

However, with RPFA on milk, the values observed in our studies were rather close to those observed with cheese in the European project. The precision values, calculated from the European studies do not seem out of reach of national or local laboratories.

↳ CONCLUSIONS

The conclusions of the European Project found both methods satisfactory and gave the following recommendations to CEN and ISO -they were all accepted in the meanwhile- :

- to amend the standards with the performance characteristics calculated in the study, following project ISO 16140 ,
- to give the same status to both parts of the standard
- to study again the description of typical, atypical and non-staphylococci colonies in ISO 6888-1.

In France, some organizations concerned by food hygiene suggested changes in the microbiological criteria for staphylococci in dairy products.

The list of abbreviations and bibliographic references are in « La Lettre de CECALAIT »

INTERESTING RECENT EU REGULATION

Commission Directive 2000/51/EC of 26 July 2000 amending Directive 95/31/EC laying down **specific criteria of purity concerning sweeteners** for use in foodstuffs (JOL 198 of 2000/8/4).

Commission Directive 2000/58/EC of 22 September 2000 amending the Annexes to Council Directives…. 86/363/EEC….on the fixing of maximum levels for pesticide residues in ….foodstuffs of animal origin…..(JOL 244 of 2000/9/29).

➢ As usual, regulation n° 2377/90, of the Council concerning maximum residue limits of veterinary drugs in foods of animal origin, has been amended.

• Annexes I, II and III were amended by **regulations 2338/2000 and 2391/2000** of 2000/10/20 and 27 (JOL 269 of 2000/10/21 and L 276 of 2000/10/28)

• Annexes I and III were amended by **regulation 1960/2000 du 15/9/2000** (JOL 234 of 2000/9/16)

• Annex I was amended by **regulation 2535/2000 of 2000/11/17** (JOL 291 of 2000/11/18)

➢ also issued

Commission Directive 2000/63/EC of 5 October 2000 amending Directive 96/77/EC laying down specific purity criteria on food additives other than colours and sweeteners

NEW

As mentioned in our latest issue, the Commission proposed a radical evolution of food safety hygiene rules. Till then, the first Community preparatory acts concerning the revision of all relevant directives were issued. The new regulation should be applicable in 2004

• Proposal for a Regulation of the European Parliament and of the Council on the hygiene of foodstuffs
http://europa.eu.int/eur-lex/fr/com/dat/2000/en_500PC0438_01.html

• Proposal for a Regulation of the European Parliament and of the Council laying down specific hygiene rules for food of animal origin.
http://europa.eu.int/eur-lex/en/com/dat/2000/en_500PC0438_02.html

• Proposal for a Regulation of the European Parliament and of the Council laying down detailed rules for the organisation of official controls on products of animal origin intended for human consumption.
http://europa.eu.int/eur-lex/en/com/dat/2000/en_500PC0438_03.html

• Proposal for a Council Regulation laying down the animal-health rules governing the production, placing on the market and importation of products of animal origin intended for human consumption.
http://europa.eu.int/eur-lex/en/com/dat/2000/en500PC0 438 _04.html

• Proposal for a Directive of the European Parliament and of the Council repealing certain Directives on the hygiene of foodstuffs and the health conditions for the production and placing on the market of certain products of animal origin intended for human consumption, and amending Directives 89/662/EEC and 91/67/EEC.
http://europa.eu.int/eur-lex/en/com/dat/2000/en_500PC0438_05.html

Official Journals of the European Communities of the last 45 days may be consulted on Internet http://europa.eu.int/eur-lex
Older texts may be ordered on Internet http://www.eudor.com

In memoriam

Rémy GRAPPIN died on 19 october 2000 at the age of 61

In 1964, he began to work as a research engineer at the INRA Station de Recherche en Technologie Laitière, here in Poligny. He became its Director in 1984 and remained until he had to stop for health reasons in 1999. He was deeply involved in the beginnings of milk payment schemes in France and since then was interested in quality control of dairy laboratories. His research interests centred on dairy analytical techniques and their precision, but also on cheese technology. He felt the importance of standardization very early and therefore was deeply involved in IDF's activities, where he was president of the Commission of analysis methods and of several groups of experts.

He was one of the fathers of CECALAIT, we miss him and we shall keep him in our memory.