Analytical capacities of
National Reference Laboratories (NRLs) and
Official Laboratories (OFLs)
in case of dioxin incidents in the feed and food chain and
conclusions for management in crisis situations

1. Introduction

After several incidents with dioxin contamination in the food chain in the past (latest incident: the contamination of Irish pork/beef at the end of 2008), it is time to draw lessons for possible future dioxin contamination incidents. Therefore, the Commission asked the CRL/NRL network to work out a **document providing advice on the time necessary for analysis of feed or food samples for PCDD/Fs and dioxin-like PCBs in case of contamination incidents and the number of samples which can be analysed within a certain time span (e.g. within one week or two weeks)**.

The **ultimate aim is to be able to lay down in a guideline for efficient and effective management of a contamination incident the maximum time period which should be observed between sampling and report of the analytical result**. Not every NRL needs to be able to accomplish this within the set period and a country faced with the situation will need to take the appropriate decisions and use laboratories which can comply with the set time period.

A detailed view should cover the following aspects

- the usefulness of the use of CALUX assays and the time needed to obtain (i) first results from use as screening method and (ii) confirmed results of suspected samples (eventually differentiated for different matrices : feed materials, compound feed, meat, milk, eggs, ...) in case of a contamination incident;

- the time needed to get a reliable result using GC/HRMS methods (eventually differentiated for different matrices: feed materials, compound feed, meat, milk, eggs, ...) in case of a contamination incident;

- the usefulness of animal biopsy samples to verify compliance wit EU legislation (publication from Philippe Marchand at Dioxin 2008);

- any other useful information related to the sampling and analysis to manage effectively and efficiently a major dioxin contamination incident.

2. Overview on analytical capabilities and discussion of different organizational structures in the EU Member States

All Member States have nominated at least one NRL. Some Member States selected two NRLs, one Member State three NRLs. As a result, NRLs may have different responsibilities:

- for determination of dioxins and/or dioxin-like PCBs and/or indicator PCBs,

- for feed and/or food,

- for confirmatory methods (applying HRMS methods) and/or GC/MS screening methods and/or bioassay screening methods.

The availability of validated methods varies very much among different NRLs.

Therefore, as a starting point, the CRL developed two questionnaires on analytical capabilities and analytical methods. The NRLs were asked to send the questionnaires to the OFLs of their countries, as well. The questionnaires differentiated between

- Use of confirmatory methods for PCDD/F and dl-PCBs (= HRMS methods),

- Use of GC/MS screening methods for PCDD/F and dl-PCBs (= LRMS methods),

- Use of bioassay screening methods for PCDD/F and dl-PCBs.

According to the answers,

- **table 1** lists the **NRLs and OFLs for confirmatory methods**,

- one NRL (Hungary: Central Agricultural Office / Central Feed Investigation Laboratory) applies **GC/MS as screening method** for PCDD/F and dl-PCBs,

- **table 2** lists the **NRLs and OFLs performing bioassay screening methods**.

Community Reference Laboratory
for Dioxins and PCBs in Feed and Food
State Institute for Chemical and Veterinary Analysis of Food, Freiburg, Germany

Country no	Country	Laboratory	NRL	OFL
1	Austria	Federal Environment Agency	X	
2	Belgium	CART	X	
	Belgium	Scientific Institute of Public Health	X	
	Belgium	Vlaamse Instelling voor Technologisch Onderzoek (VITO)		X
3	Bulgaria	National Veterinary Service	X	
4	Cyprus 1)	Food: State General Laboratory (SGL), Pesticide Residues Laboratory	X	
	Cyprus 1)	Feed: Oekometric	X	
5	Czech Republic	Inst. Pub. Health Ostrava (Zdravotni ustav)	X	
	Czech Republic	ALS Czech Republic, s.r.o.		X
	Czech Republic	Axys - Varilab s.r.o, 252 46 Vrané nad Vltavou, Vltavská 13		X
	Czech Republic	State Veterinary Institute Prague		X
6	Denmark	National Food Institute, Technical University of Denmark	X	
	Denmark	Danish Veterinay and Food Administration, Reagion East, Ringsted		X
7	Estonia	Tartu Laboratory of Public Health Inspectorate	X	
8	Finland	Chemical Exposure Unit, National Institute for Health and Welfare	X	
9	France 2)	LABERCA	X	
	France	CARSO-LSEHL		X
10	Germany	BfR	X	
	Germany	Landeslabor Berlin-Brandenburg		X
	Germany	LUA Sachsen, Dresden		X
	Germany	LUFA Speyer		X
	Germany	LUFA Rostock		X
	Germany	Landesuntersuchungsamt, Institut für Lebensmittelchemie Speyer		X
	Germany	Lower Saxony Federal State Office for Consumer Protection and Food Safety (LAVES)		X
	Germany	CVUA Freiburg		X
	Germany	CVUA Münster		X
11	Greece 3)	Mass Spectrometry and Dioxin Analysis Lab. NCSR "Demokritos"	X	
12	Hungary	Central Agricultural Office Food and Feed Safety Directorate, FEED_NRL	X	
	Hungary	Hungarian Central Agricultural Office, Central Food Toxicological Laboratory	X	

Community Reference Laboratory
for Dioxins and PCBs in Feed and Food
State Institute for Chemical and Veterinary Analysis of Food, Freiburg, Germany

13	Ireland	The State Laboratory	X	
14	Italy	Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale"	X	
	Italy	Istituto zooprofilattico sperimentale Lombardia Emilia Romagna -Bologna		X
15	Latvia	National Diagnostic Centre	X	
16	Lithuania	National Food and veterinary Risk Assessment Institute	X	
17	Luxembourg	Oekometric	X	
18	Malta	CSL 5)	X	
19	Poland	National Veterinary Research Institute (NVRI) PULAWY	X	
20	Portugal	INETI/LAQAS	X	
21	Romania	Institute for Hygiene and Veterinary Public Health	X	
22	Slovakia	National Reference Centre for Dioxins and Related Compounds	X	
23	Slovenia	Public Health Institute Maribor	X	
24	Spain	CNA	X	
25	Sweden 4)	Eurofins	GfA GmbH		X
26	The Netherlands	RIKILT	X	
27	UK (and Malta)	Central Science Laboratory 5)	X	

1) The NRL for food contracted the Austrian NRL as laboratory for performance of confirmatory analyses). For feed , Oekometric was contracted.

2) LABERCA for dioxins and dl-PCBs (here included); AFFSA for ndl-PCBs (here not included)

3) NCSR for dioxins and all PCBs (here included); Institute of Food Hygiene of Athens for ndl-PCBs (here not included)

4) Eurofins as contract laboratory for NRL

5) no routine analyses - only projects

Table 1: List of NRLs and OFLs for confirmatory methods for determination of dioxins and dioxin-like PCBs in EU Member States

Country	Laboratory	NRL	OFL
Belgium	Federal laboratory for the Safety of the Foodchai	X	
Belgium	Scientific Institute of Public Health	X	
Cyprus	State Genral Laboratory (SGL)	X	
Germany	Lower Saxony Federal State Office for Consume		X
Poland	NVRI PULAWY	X	
Slovakia	State Veterinary and Food Institute Kosice	X	
The Netherlands	RIKILT	X	

Table 2: List of NRLs and OFLs applying bioassay screening methods for determination of dioxins and dioxin-like PCBs in EU Member States (all NRLs established CALUX-based methods, whereas the German OFL introduced EROD for validation purposes within a project).

For calculation of the available analytical capabilities in each Member State and of the overall sum, a differentiated view on the **organizational structures in the EU Member States** is necessary for a better understanding:

1. Inclusion of NRLs and OFLs for calculation of the available capacity in each Member State: In a crisis situation, official samples can be analyzed by NRLs and/or OFLs. Therefore, the analytical capacities of each Member State are characterized by the sum capacity of the NRL (or all NRLs, if more than one NRL has been established) and all OFLs (if existing).

2. The following countries have two NRLs for determination of dioxins and dioxin-like PCBs with confirmatory methods: Belgium; Hungary and Cyprus.

3. The following countries have NRLs with bioassay screening methods: Belgium, Cyprus, Poland, Slovakia and The Netherlands (see table 2). No country has bioassay capacities without capabilities for GC/HRMS confirmation.

4. The following countries have OFLs for confirmatory methods in addition to NRLs: Belgium, Czech Republic, Denmark, France, Germany, Italy, and Sweden.

5. All NRLs established CAL^^UX-based methods, whereas the German OFL introduced EROD for validation purposes within a project and does not use this test for official control.

6. One private laboratory was contracted as NRL or OFL by two countries: Oekometric (Bayreuth) as NRL for feed in Cyprus and for all tasks for Luxembourg. Eurofins GfA Hamburg was contracted as official OFL for Sweden and performed analyses (without official OFL status) for Malta. The capacities of these laboratories are counted only once when calculating the overall sum for all nominated labs.

Community Reference Laboratory
for Dioxins and PCBs in Feed and Food
State Institute for Chemical and Veterinary Analysis of Food, Freiburg, Germany

3. Analytical capacities for confirmatory methods

Table 3 gives an overview on the **number of samples routinely analysed per year by the laboratories listed in table 1 (using confirmatory methods** for determination of dioxins and dioxin-like PCBs in feed and food). Results are:

- 23 Member States have laboratories routinely analyzing food for dioxins and 21 Member States have laboratories routinely analyzing feed samples.

- About 18,200 food samples and 15,000 feed samples are routinely analysed per year.

- If the analyses performed by these laboratories are summed up for each Member State, 250 samples would be routinely analysed as median, with a range between 20 and 5000 for food respectively 5 and 5000 for feed.

- However, these figures cannot be taken as samples which are really analysed routinely for the Member States: Table 3 comprises two private laboratories analyzing between 2500 and 5000 food respectively feed samples per year. Only few of these samples are assumed to have been analyzed for the countries having selected these laboratories.

Statistical data	Food	Feed
No of countries with analytical capacities for confirmatory methods	22	21
Sum of routinely analysed samples per year	18195	14980
Average available capacity per country		
Min	20	5
25 %-Percentile	68	58
Median	250	250
MW	866	788
75 %-Percentile	830	600
Max	5000	5000

Table 3: Number of samples routinely analysed with confirmatory methods per year by the laboratories listed in table 1 and range of analytical capacity of these laboratories

Therefore, the discussion of the results presented in table 3 results in an important difference between NRLs or OFLs performing their tasks as mainly official control authority and private laboratories working as NRL or OFL or cooperating with NRLs. As an example, laboratories working for the official food control in Germany are not allowed to analyse

Community Reference Laboratory
for Dioxins and PCBs in Feed and Food
State Institute for Chemical and Veterinary Analysis of Food, Freiburg, Germany

commercially for Third Parties: The concept is that analyses for self-control of the industry are performed by private laboratories and that the official control should "only" control this self-control. Therefore, private laboratories were established offering considerable analytical capacities (several thousand samples per year) for analyses of dioxins in feed and food, whereas the official control laboratories - run by the Federal States (Bundesländer) - are set up to run samples in the order of several hundred samples per year to perform the public control.

Table 4 presents the **weekly analytical capacities for confirmatory methods for determination of dioxins** (as WHO-PCDD/F-TEQ, without inclusion of dioxin-like PCBs).

Control of dioxins without dl-PCBs	Food					Feedingstuff		
	Milk	Meat	Eggs	Fish	Others	Compound feed	Additives	Others
No of countries with analytical capacities for confirmatory methods	18	18	18	19	15	18	17	11
Sum of weekly capacity	1016	995	884	1098	930	1195	1185	821
Average available capacity per country								
Min	3	3	3	2	6	10	10	6
25 %-Percentile	14	14	14	10	20	15	18	30
Median	30	30	30	21	35	35	40	40
MW	60	59	52	61	66	75	79	91
75 %-Percentile	72	80	80	58	75	88	96	80
Max	300	300	150	400	300	400	400	400

Table 4: Number of samples which can be analysed within one week for determination of dioxins (without dioxin-like PCBs) with confirmatory methods by the laboratories listed in table 1 and range of available analytical capacity per Member State

Up to 19 Member States reported the availability of analytical capacity for determination of dioxins in the requested food or feed matrices. As a sum for all Member States, about 900 - 1100 food samples or about 800 - 1200 feed samples can be analysed per week. The matrix has a limited effect: The number of samples depends on the type of food samples: Whereas about 880 egg samples could be analysed within a week, for fish the number would increase to about 1100. (The numbers have to be understood as weekly capacity in a crisis situation for milk or meat or eggs etc.). Again, a wide range of analytical capability

would be available. The maximum number would be reduced if the private laboratory contracted by a Member State would have to split up its capacity for several clients (e.g. the two Member States according to the list in table 1 and possibly additional clients).

The request from the Commission and to questionnaire sent to the NRLs/OFLs did not specify a definition of a "week". Therefore, it is assumed that "a week" was understood generally as 5 working days plus Saturday / Sunday. This offers the opportunity to increase the capacity if for a limited period (depending on the dimension of the crisis or incident) under an emergency situation laboratory work is performed also on weekends.

Table 5 presents the **weekly analytical capacities for confirmatory methods for determination of dioxins and dioxin-like PCBs**. The numbers are comparable to table 4. As a result, there is no big difference between analytical capabilities for determination of dioxins, only, or for separate determination of dioxins and dioxin-like PCBs using confirmatory methods.

dioxins + dl-PCBs	Food					Feedingstuff		
	Milk	Meat	Eggs	Fish	Others	Compound feed	Additives	Others
No of countries with analytical capacities for confirmatory methods	18	18	18	18	14	17	15	10
Sum of weekly capacity	949	945	832	1046	865	1137	1142	806
Average available capacity per country								
Min	3	3	3	3	6	10	10	0
25 %-Percentile	14	10	12	12	17	15	15	18
Median	30	30	30	17	38	40	40	35
MW	56	56	49	62	67	76	76	81
75 %-Percentile	70	80	80	55	75	88	88	73
Max	300	300	150	400	300	400	400	400

Table 5: Number of samples which can be analysed within one week for determination of dioxins and dioxin-like PCBs with confirmatory methods by the laboratories listed in table 1 and range of available analytical capacity per Member State

Table 6 presents the **analytical capacities for confirmatory methods for determination of dioxins and dioxin-like PCBs for a period of four weeks**. The numbers are quite linear to the extension of time in comparison to table 5. As a result, between about 3200 and 4400 food samples or between 3000 and 4500 feed samples could be analysed by all laboratories applying confirmatory methods for determination of dioxins and dioxin-like PCBs. Again, it is important to mention that these maximum numbers include private laboratories whose capacities probably would be split between different clients.

dioxins + dl-PCB	Food					Feedingstuff		
	Milk	Meat	Eggs	Fish	Others	Compound feed	Additives	Others
No of countries with analytical capacities for confirmatory methods	19	18	19	19	13	16	16	10
Sum capacity for 4 weeks	4102	3801	3691	4401	3238	4439	4464	3062
Average available capacity per country								
Min	15	15	15	15	50	35	35	72
25 %-Percentile	57	56	56	40	86	86	86	98
Median	91	90	91	91	120	140	145	150
MW	228	224	205	245	270	317	319	383
75 %-Percentile	258	260	258	225	281	389	389	355
Max	1500	1200	900	1600	1200	1600	1600	1600

Table 6: Number of samples which can be analysed within four weeks for determination of dioxins and dioxin-like PCBs with confirmatory methods by the laboratories listed in table 1 and range of available analytical capacity per Member State

The analytical procedures can be performed **manually** or **automated**. Modern automated analyses comprise:
  ➢ Automated extraction (e.g. ASE [accelerated solvent extraction] or PLE [pressurized liquid extraction]);
  ➢ Automated clean-up (e.g. Power Prep[TM] [Fluid Management System, FMS]).

At this point is has to be made clear that the CRL / NRL network does not endorse any commercial system or company. Therefore, the reference to any company or product in this working document does not express any preference or recommendation but is meant solely as example for technical solutions.

Advantages and disadvantages are:

- **Manual** procedures:
  - mostly lower costs for chemicals;
  - considerably lower costs for technical equipment;
  - higher costs for staff (due to longer time for analysis);
  - time-consuming (e.g. up to a week for freeze-drying, extraction and clean-up).

- **Automated** procedures:
  - Can be very fast, if extraction (PLE / ASE) and clean-up is automated (e.g. one day for extraction and clean-up)
  - Higher costs for chemicals for automated clean-up (complete chromatographic columns for one use, only).
  - Higher instrumental skills of technicians required.

The decisive advantage of automated procedures with regard to time for analyses becomes obvious when comparing the analytical methods established at the CRL.

- The **routine method based on manual procedures** comprises the following steps:
  - Sample preparation (homogenization and preparation of a representative aliquot);
  - Freezing (over night) and freeze-drying (about 1 day for 8 samples simultaneously of up to 200 - 300 g per sample);
  - Extraction (Twisselmann hot extraction) and evaporation of the extract: less than a day;
  - Gelchromatography for separation of dioxins and PCBs from 3 g fat: about 3 hours per sample (4 injections); about 24 hours for 8 samples; followed by evaporation of the fat-free extract;
  - Manual clean up-steps (small silica / sulfuric acid column; florisil column) (including evaporation): one working day;
  - Automated clean up on activated carbon (Carbopack) separately for dioxins and PCBs, including evaporation and preparation of the final extracts for GC/HRMS determination: one day.

  As a result, roughly one week is necessary to obtain extracts ready for GC/MS determination. One technician can run two sequences each of e.g. up to 10 samples time-delayed: Even if the individual steps are time-consuming, that does not mean that the technician is kept busy during this time: The real working time for freezing, freeze-drying, extraction, gelchromatography and the carbon clean up is only a small part of the overall time.

- As examples for **automated procedures**, extraction by ASE and PLE and the automated clean up system "PowerPrep" (FMS) have been introduced. These

systems were used when in December 2008, the CRL was asked by the Polish NRL for Dioxins and PCBs for the confirmation of extremely high concentrations for PCDD/Fs in samples of bacon and pork liver from Poland related to the Irish meat incident. The samples were analyzed in single analysis according to validated method for the analysis for PCDD/Fs and PCBs in meat at the CVUA Freiburg using ASE extraction and PowerPrep clean-up. The results of the Polish NRL were confirmed for all three samples and reported to Poland within less than four days (first shipment of samples) respectively less than three days (second shipment of samples). The individual steps comprised:

➢ Sample preparation (homogenization and preparation of a representative aliquot);

➢ Mixing with adsorbent for binding of water and ASE extraction over night;

➢ FMS fully automated clean up (system with units for parallel treatment of samples): less than a day for evaporation of the extracts, clean up, evaporation of the purified extract and preparation of the final extract for GC/HRMS determination.

As a result, when samples are available in the morning of day 1, the samples can be ready for extraction over night and the extracts be purified the following day allowing GC/HRMS determination the next night. The number of samples which can be handled in parallel depends on the available equipment (in particular number of units for parallel FMS clean-up) and the number of technicians. The FMS-system can be equipped with up to 6 units for simultaneous analysis. With such a system, two runs can be performed within one working day per technician.

FMS has introduced a new system including automated evaporation after PLE extraction and after clean up. In combination with large-volume injection for GC/HRMS detection, this system promises the best available technique for automated fast analyses. This system was presented by Jef Focant (University of Liège, Belgium) at the "3rd European High Resolution GC/MS Users Meeting" (March 26 and 27, 2009, Rome, Italy): "FMS Total Sample Preparation and Thermo Scientific Large Volume Injection". The system is described as follows: "TRP Total-Rapid-Prep™" (Integrated Extraction, Sample Clean-up and Concentration System):

FMS introduces the first and only "Total Solution" Sample Prep system available that combines three sample prep processes into one economical package. The TRP Total-Rapid-Prep performs the extraction, sample clean-up and evaporation for six samples simultaneously in less than a few hours, producing the highest recoveries and best results for all analytes. A Windows based system that is easy to use, controls the system and is fully programmable. With the TRP system one has the option to run a single sample prep process such as extraction, sample cleanup or evaporation. One may also run a variety of processes including extraction, clean-up and evaporation in a single step. The TRP system increases sample throughput while reducing errors and poor recoveries. It also provides a cleaner background and eliminates cross contamination due to it's advanced closed loop system design. TRP uses FMS's high

quality and inexpensive Teflon based prepacked disposable columns which guarantee high recoveries and eliminate glassware clean-up. Applications include Dioxins, PCBs, PAHs, PBDEs and Pesticides.

An unanswered question, so far, is how extracts can be completely re-dissolved after evaporation by this technique, in particular, if solvent change is necessary e.g. after extraction with proportions of polar solvents and re-dissolving with an unpolar solvent. Therefore, some questions have to be answered before the fully automated analyses will be fully validated. Nevertheless, the most important individual steps (automated extraction and automated clean up) are available and can be validated by the applying laboratory separately, and different evaporation techniques and strategies to re-dissolve the extracts are available to take advantage of these time-saving analytical options.

As a result, confirmatory methods

> have optimal quality control possibilities (control of 13C-labelled internal standards for each of the congeners of interest and in this way control of any losses of dioxins or dioxin-like PCBs for every individual sample during analysis);

> have low limits of quantification (LOQs) for the congeners allowing reliable analysis of samples also at low levels (e.g. control of low action levels),

> allow determination of the individual congeners and in this way allow recognition and discussion of congener patterns (traceability of samples to assumed source);

> include separate determination of dioxins and dioxin-like PCBs;

> can be fully automated for high sample-throughput and short time for analysis.

4.   Analytical capacities for screening methods

Table 7 gives an overview on the **number of samples routinely analysed per year by the laboratories listed in table 2 (using bioassay screening methods** for determination of dioxins and dioxin-like PCBs in feed and food). Results are:

- 5 Member States have laboratories routinely analyzing food for dioxins and dioxin-like PCBs and 4 Member States have laboratories routinely analyzing feed samples.

- About 1,800 food samples and 1,100 feed samples are routinely analysed per year.

- If the analyses performed by these laboratories are summed up for each Member State, about 150 food and 260 feed samples would be routinely analysed as median, with a range between 20 and 1140 respectively 30 and 500.

	Food	Feedingstuff
No of countries with analytical capacities for bioassay screening methods	5	4
Sum of samples routinely analysed per year:	1834	1069
Average capacity per country with bioassay capacities:		
Min	20	30
25 %-Percentile	120	98
Median	153	260
MW	367	267
75 %-Percentile	400	430
Max	1141	519

Table 7: Number of samples routinely analysed with bioassay screening methods per year by the laboratories listed in table 1 and range of analytical capacity of these laboratories

Bioassays determine a sum parameter which is best expressed as **"bioanalytical equivalents"** (**BEQ**). BEQ can comprise either the sum parameter for the sum of dioxins, furans and dl-PCBs or the separately determined BEQs for (i) dioxins and furans and (ii) dl-PCBs. BEQ values have to be comparable to TEQ-based legislation: The results may be expressed as TEQ, thereby assuming that the compounds responsible for the signal correspond to the TEQ principle.

Table 8 gives the number of samples which can be analysed within one week by bioassay screening methods for dioxins (without dioxin-like PCBs), **table 9** for separate determination of dioxins and dioxin-like PCBs within one week and **table 10** within four weeks.

When comparing table 8 and 9 in detail, it becomes obvious that the maxima are the same whereas the other data are different. These observations can be explained by the different numbers of laboratories included in these two tables and as result of the different approaches for bioassay screening:

- The laboratory with the highest capacity has established a routine method which includes the separation of dioxins and dioxin-like PCBs. Therefore, the analytical capacity for determination of the sum parameter or of the separate parameters is identical.

- The laboratory with the second highest capacity has established a routine method which determines the sum parameter for the sum of dioxins and dioxin-like PCBs in one (after clean-up re-dissolved final) extract. If dioxins and dioxin-like PCBs are to be determined separately, then an additional clean-up step has to be added and the two fractions have to be determined separately. This reduces the analytical capacity.

	Milk	Meat	Eggs	Fish	Others	Compound feed	Additives	Others
No of countries with analytical capacities for bioassay screening methods	3	2	3	3	1	4	2	1
Sum of samples analyzable for WHO-PCDD/F-TEQ within 1 week	273	310	273	336	26	353	300	100
Average capacity per laboratorywith bioassay capacities:								
Min	13	60	13	26	26	13	100	100
25 %-Percentile	37	108	37	43	26	33	125	100
Median	60	155	60	60	26	70	150	100
MW	91	155	91	112	26	88	150	100
75 %-Percentile	130	203	130	155	26	125	175	100
Max	200	250	200	250	26	200	200	100

Table 8: Number of samples which can be analysed within one week for determination of dioxins (without dioxin-like PCBs) with bioassay methods by the laboratories listed in table 1 and range of available analytical capacity per laboratory

	Milk	Meat	Eggs	Fish	Others	Compound feed	Additives	Others
No of countries with analytical capacities for bioassay screening methods	5	3	4	3	2	4	3	1
Sum of samples analyzable for WHO-PCDD/F-TEQ and WHO-PCB-TEQ within 1 week	286	320	273	320	36	350	310	100
Average capacity per laboratorywith bioassay capacities:								
Min	3	10	3	10	10	10	10	100
25 %-Percentile	10	35	8	35	14	33	55	100
Median	13	60	35	60	18	70	100	100
MW	57	107	68	107	18	88	103	100
75 %-Percentile	60	155	95	155	22	125	150	100
Max	200	250	200	250	26	200	200	100

Table 9: Number of samples which can be analysed within one week for separate determination of dioxins and dioxin-like PCBs with bioassay methods by the laboratories listed in table 1 and range of available analytical capacity per laboratory

	Milk	Meat	Eggs	Fish	Others	Compound feed	Additives	Others
No of countries with analytical capacities for bioassay screening methods	5	4	3	3	2	3	3	1
Sum of samples analyzable for WHO-PCDD/F-TEQ and WHO-PCB-TEQ within 4 weeks	1270	1390	1130	1330	134	1330	1330	500
Average capacity per laboratorywith bioassay capacities:								
Min	30	30	30	30	30	30	30	500
25 %-Percentile	60	53	165	165	49	265	265	500
Median	80	180	300	300	67	500	500	500
MW	254	348	377	443	67	443	443	500
75 %-Percentile	300	475	550	650	86	650	650	500
Max	800	1000	800	1000	104	800	800	500

Table 10: Number of samples which can be analysed within four weeks for separate determination of dioxins and dioxin-like PCBs with bioassay methods by the laboratories listed in table 1 and range of available analytical capacity per laboratory

As a result, bioassay screening methods

- ➢ can be applied with high sample-throughput and short time for analysis;

- ➢ are established with two different approaches in the NRL network:

  - ✓ routinely separate determination of dioxins and dioxin-like PCBs or

  - ✓ routine determination of the sum parameter (WHO-PCDD/F-PCB-TEQ) as fastest method with the possibility to add an additional step for separate determination of dioxins and dioxin-like PCBs (which then is more time-consuming and reduces the analytical capacity).

5.   Lessons from the Mozzarella Incident (Italy, 2008):

This chapter is based on the following information:

- ✓ Silvio Borrello, Gianfranco Brambilla, Loredana Candela, Gianfranco Diletti, Pasquale Gallo, Nicola Iacovella, Giuseppe Iovane, Antonio Limone, Giacomo Migliorati, Ornella Pinto, Paolo Sarnelli, Luigi Serpe, Giampiero Scortichini, and Alessandro di Domenico "Management of the 2008 "Buffalo Milk Crisis" in the Campania Region under the Perspective of Consumer Protection", Organohalogen Compounds, Volume 70 (2008) page 000892 (presented at Dioxin 2008 in Birmingham, UK)

- ✓ Giampiero Scortichini (National Reference Laboratory for PCDDs, PCDFs, and DL-PCBs in Feed and Food, 64100 Teramo, Italy) "Dioxin Monitoring in Food and Feed in Italy: Contamination Incidents in the Period 2002 – 2008", presentation at 3rd European High Resolution GC/MS Users Meeting, March 26 and 27, 2009, Rome, Italy

- ✓ Alessandro di Domenico (Italian National Institute for Health, Rome), presentation at "Dioxin: Environment and Health", 29 - 30 April 2009, Naples, Italy

- ✓ Giampiero Scortichini (National Reference Laboratory for PCDDs, PCDFs, and DL-PCBs in Feed and Food, 64100 Teramo, Italy) "Results of an extraordinary monitoring plan 2008 - 2009", information provided to the CRL

In mozzarella of the Campania Region (Italy), a significant part of the cheese samples collected during a monitoring programme performed in 2007 / 2008 proved to be non-compliant with EU regulation for dioxins and dioxin-like PCBs. The production was confined to a limited area of the region. In particular, inappropriate waste disposal may have been a relevant cause of the problems, although its importance has still to be clarified. The European Commission (EC) asked the Italian government to warrant the safety of the food products (in particular mozzarella) with the adoption of an extraordinary monitoring plan, to be applied to the entire region. Results should be provided within one month, as a preventive measure to limit a possible export ban on the product.

With this background, the European Commission needed emergency assistance asking the CRL to provide within few hours an overview on the analytical capacity (CALUX- or GC/HRMS-based methods) of laboratories in the EU for performing analysis on dioxins (and dioxin-like PCBs) in milk and dairy products from the Campania region for a third party and the approximate price for an analysis (screening and confirmatory tests). The overview should concern samples for which the analysis have to be performed in one weeks time.

The list finally provided to Italy comprised 5 laboratories performing confirmatory methods offering 400 analyses per week (with separate determination of dioxins and dioxin-like PCBs) and 3 labs performing CALUX screening methods offering 650 analyses per week (without separate determination of dioxins and dioxin-like PCBs). One CALUX lab offered a considerably reduced number of analyses for separate determination of dioxins and dioxin-like PCBs.

On this basis, the required comprehensive tests could be performed for the requested control programme in Italy. With regard to unanswered questions related to the source of contamination, the Italian authorities chose as strategy to analyze for dioxins and for dioxin-like PCBs separately and to take the EU-action limits (2 pg WHO-PCDD/F-TEQ/g fat and 2 pg WHO-PCB-TEQ/g fat) as reference point to decide on acceptability of the samples. The Commission accepted this approach.

According to the Regulation (EC) 1883/2006, *monitoring for the presence of dioxins in foodstuffs may be performed by a strategy involving a screening method in order to select those samples with levels of dioxins and dioxin-like PCBs that are less than 25 % below or exceed the maximum level. The concentration of dioxins and sum of dioxins and dioxin-like PCBs in those samples with significant levels needs to be determined/confirmed by a confirmatory method.*

The monitoring plan was divided into three phases:
- Phase I: analysis of milk samples taken in the dairy factories located in the provinces of Avellino, Caserta and Naples (high frequency of non-compliant samples expected);
- Phase II: analysis of milk samples taken in the dairy factories located in the provinces of Salerno and Benevento (low frequency of non-compliant samples expected);
- Phase III: analysis of milk and feed samples taken in the dairy farms traced back according to the non-compliant results obtained in the Phase I and Phase II. In addition, buffalo, bovine and sheep/goats livestock located in the area of 3 km from the centre of a "positive" farm were controlled.

The Phase I and Phase II had to completed in 1 month. Taking into account the large number of samples to be analysed, it was decided to adopt a "pooled milk sampling" approach whenever applicable for a maximum of four different milk samples constituting a

pool. The chosen approach permitted to analyse 381 milk samples instead of 959 (the total number of farms delivering milk to the dairy factories subjected to the official control). As a result, the samples were made of milk produced by a single farm or by 2 – 4 farms.

As a consequence, the limit of discrimination between compliant and suspected non-compliant milk samples in a screening analysis for the determination of the **total toxic equivalents (PCDD/Fs + dl-PCBs)** was set at **2.25 pg WHO-TEQ/g fat for individual milk samples** and at **1.50 pg WHO-TEQ/g fat for pooled milk samples**.

All samples were analysed by HRGC/HRMS. The required measurement uncertainty was < ±20 %.

The Italian authorities used the analytical capacities of the NRL and the Italian OFLs and in addition chose one private laboratory performing confirmatory methods and offering a high sample throughput within one week to support the NRL and OFLs (for more details, see above mentioned publication of Silvio Borello et al, 2008).

The decision of Italy was based also on the aspect to avoid delays by confirmation of samples which could have been pre-analysed with bioassay screening methods: After bioassay screening, positive results must be confirmed by HRMS-based methods. This requires additional organization (shipment of an additional aliquot to the confirmatory laboratory), time (for confirmation) and costs. As a significant number of samples was expected to exceed the action levels, Italy decided to take only laboratories into consideration offering high sample throughput with confirmatory methods. The correctness of this assumption was confirmed by the final results:

Phase I and II (381 milk samples):

- 21 out of 67 (31.3%) individual milk samples were ≥ 2.25 pg WHO-TEQ/g fat

- 145 out of 314 (46.2%) pooled milk samples were ≥ 1.50 pg WHO-TEQ/g fat

Phase III (433 milk samples):

- 306 out of 433 (70.7%) individual milk samples were ≥ 2.25 pg WHO-TEQ/g fat

In conclusion, 166 out of 381 (43.6%) samples would have to be confirmed by HRGC-HRMS during the Phase I and Phase II (1 month time frame) if a bioassay screening had been chosen and if the bioassay screening could have proved the same measurement uncertainty. On a whole, 472 out of 814 (58.0%) samples would have to be confirmed by HRGC-HRMS following a screening approach. With higher measurement uncertainties for screening methods, this proportion would have been higher than calculated on a basis of < ±20 % for confirmatory methods.

6. Technical considerations regarding sampling and shipment of samples:

➢ A sampling strategy has to be developed and the number of samples to be in agreement with available capacity. Sampling and shipment have to be performed with the same high priority as the request to perform the analyses as soon as possible.

➢ Sampling and shipment to the laboratory has to be performed in close contact to the laboratory to make sure that samples arrive on time to allow simultaneous analyses of a number of samples and in order to avoid overloading at certain times (e.g. by uncoordinated shipment / delays in sampling)

7. Usefulness of biopsy samples to verify compliance with EU legislation

Chapter 7.1 summarizes the observations and conclusions presented by Marchand et al (Organohalogen Compounds 70 (2008) 000906 - 000909). The discussion of the general applicability of these conclusions have to take into consideration several aspects which are presented in chapters 7.2 - 7.6. For conclusions, see chapter 7.7.

7.1 Relation between dioxin concentration levels in fat, muscle and blood

Marchand et al (2008) analysed blood, muscle and four sorts of fat tissue samples to calculate the correlation between these tissues in terms of the dioxin contamination level. Peripheric kidney (= perirenal) fat was chosen as model of internal fat. The additional 3 subcutaneous fat samples were located behind the ear, near the sternum and sub-caudal, respectively. **Table 11** summarizes the obtained results.

Type of animal	Animal Identification	sub-caudal fat WHO-TEQ (pg/g fat)	Peripheric kidney fat WHO-TEQ (pg/g fat)	blood WHO-TEQ (pg/g fat)	muscle WHO-TEQ (pg/g fat)
beef cattle	5953	0.2	0.25	-	0.29
beef cattle	5957	0.25	0.2	0.98	0.29
beef cattle	2082	0.74	0.47	1.26	0.70
beef cattle	9111	0.8	0.61	1.15	0.53
beef cattle	4426	4.95	5.19	5.15	4.01
beef cattle	6372	4.97	5.86	3.75	3.55
beef cattle	9073	6.04	6.08	6.47	3.67
calf	7551	6.32	6.4	5.16	-
calf	6368	7.51	7.32	5.69	-
beef cattle	2009	8.32	9.32	7.52	-
beef cattle	2015	9.75	10.95	9.77	-
beef cattle	5304	17.82	17.12	18.85	12.08
calf	8263	20.92	22.71	21.92	17.43
beef cattle	7372	24.52	21.59	23.24	-

Table 11: WHO-PCDD/F-TEQ levels determined in muscle, fat and blood samples from the same animals (Marchand et al, 2008)

For comparison, the maximum level for dioxins in meat and meat products is as follows (Commission Regulation [EC] No 1881/2006):

- Bovine animals and sheep: 3,0 pg WHO-PCDD/F-TEQ/g fat;

- Poultry: 2,0 pg WHO-PCDD/F-TEQ/g fat;

- Pigs: 1,0 pg WHO-PCDD/F-TEQ/g fat.

If a clearly significant correlation was found between dioxin levels in perirenal fat and muscle, it was noticed that the concentration observed in muscle appears to be systematically about 30 to 40 % lower than the concentration measured in internal fat (peripheric kidney fat) (**Figure 1**).

correlation beetween muscle and internal fat

$y = 0,7351x$
$R^2 = 0,9924$
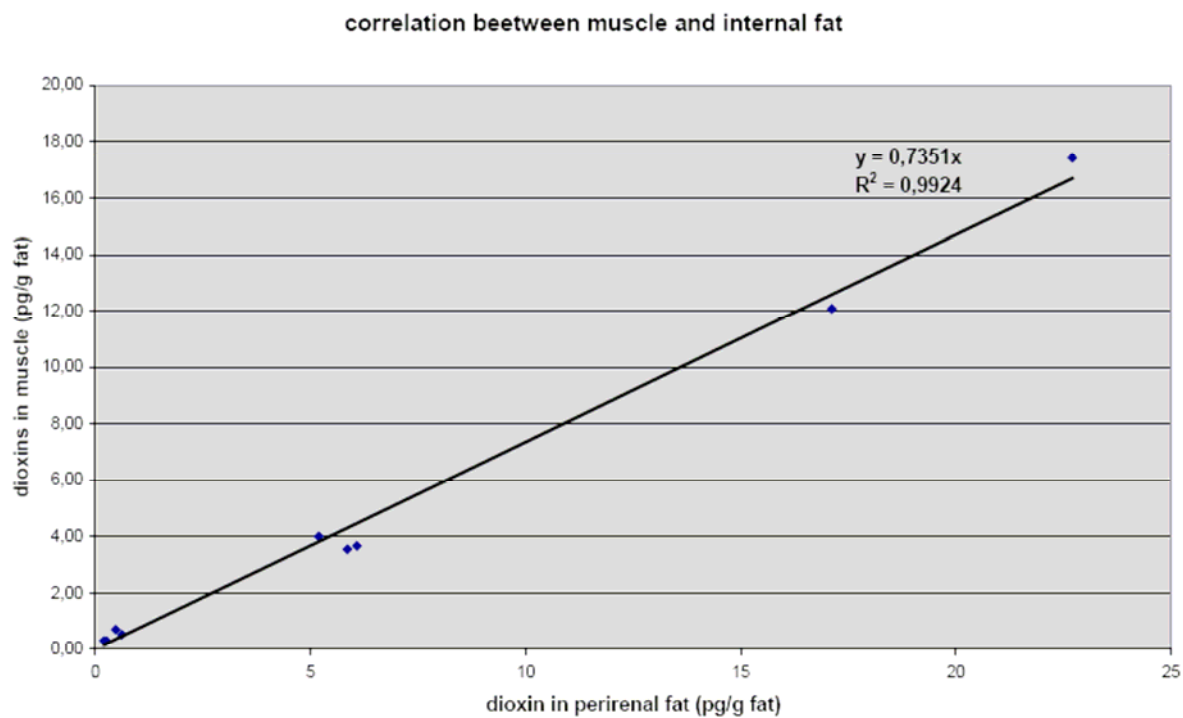
Figure 1: Correlation observed between PCDD/F concentration levels measured in muscle and peripheric kidney fat samples

Conversely, no similar systematic deviation was observed for the correlation between blood and peripheric kidney fat samples (**Figure 2**) or between internal and external fat samples (**Figure 3**).
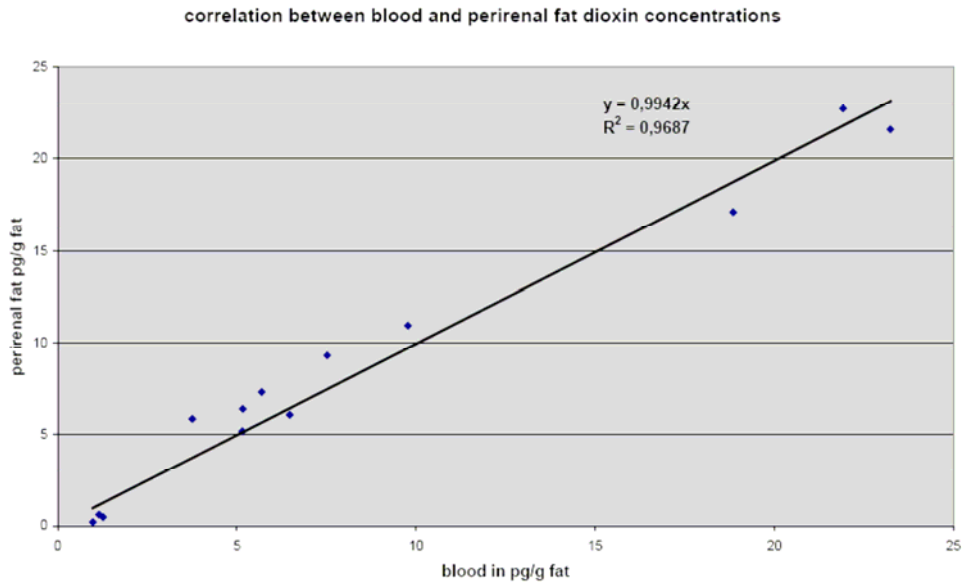
correlation between blood and perirenal fat dioxin concentrations

$y = 0,9942x$
$R^2 = 0,9687$

Figure 2: Correlation between PCDD/F concentration levels measured in blood and peripheric kidney fat samples

correlation between perirenal and sub-caudal fat

$y = 0,9869x$
$R^2 = 0,9793$
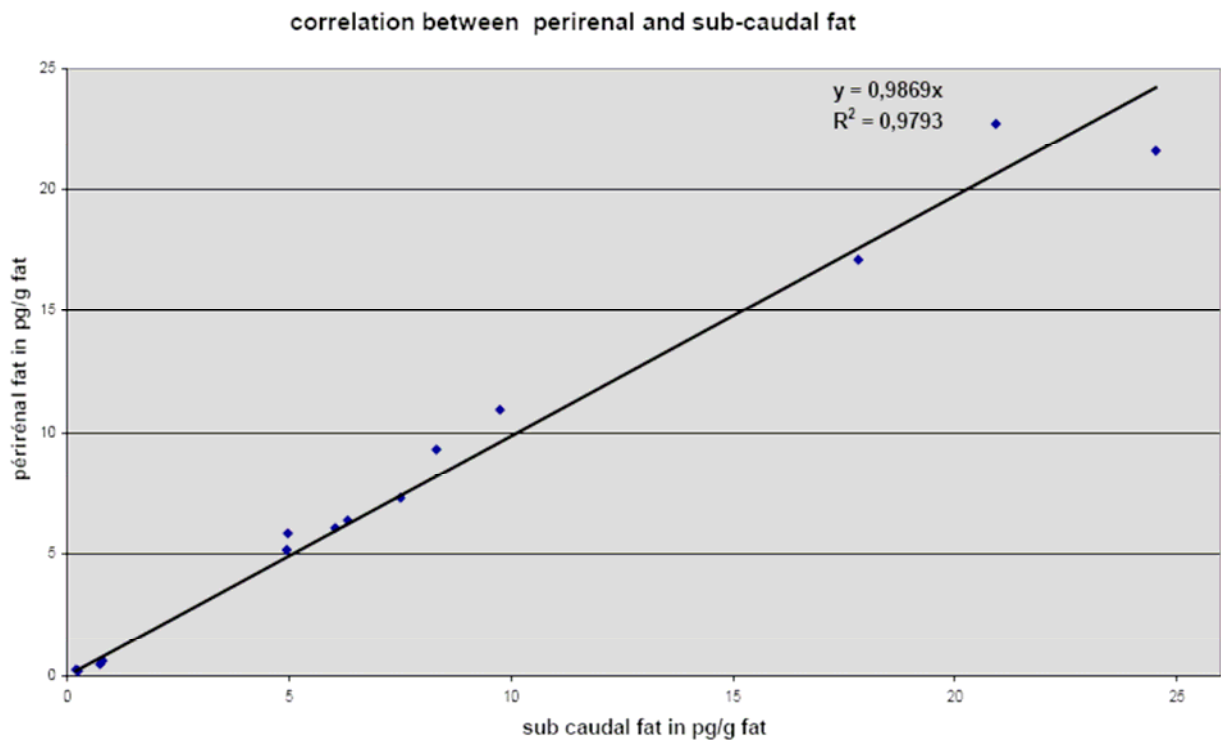
Figure 3: Correlation observed between PCDD/F concentration levels measured in peripheric kidney fat samples and subcutaneous fat samples

The authors summarize the main conclusions as follows:

 ➢ a very good correlation between the dioxins level of sub-caudal fat taken by biopsy and internal fat from the same animal was demonstrated,

 ➢ a good correlation between the results for blood and internal fat was also observed,

 ➢ no significant difference was observed in terms of PCDF/PCDD concentrations for 2 animals coming from the same farm and fed with the same alimentation.

According these results, a biopsy of fat as well as collection of blood samples was proposed as useful and efficient method to draw conclusions on the compliance of the animal.

7.2 Pharmacokinetics

Dioxins can be absorbed through the gastrointestinal tract, skin, and lungs. The degree of absorption varies with the congener, the route of absorption, and the way of intake. The oral route usually accounts for more than 90 % of the total intake for humans with background contamination. The oral intake e.g. via feed or soil is an important factor also for the intake of animals.

When dioxins are orally administered to experimental animals, they are principally distributed to the blood, liver, muscles, skin, and adipose tissue. Bioaccumulation is particularly prominent in the liver and adipose tissue. Their distribution also varies with the congeners and dose.

Dioxins are generally difficult to be metabolized. They are mainly excreted in feces. Long half-lives were observed.

Accumulation of a compound in an organism occurs whenever the time interval of exposure is small compared to the half-life. After a certain time of continuous exposure, an equilibrium (Steady-State) is reached. At steady-state, the amount of dioxins absorbed is equal to the amount of dioxins eliminated (by metabolization, transfer e.g. to eggs or milk and excretion via faeces). Carry-over rates e.g. from feed to milk can be calculated for steady-state.

If there is no steady-state, the dioxin concentrations in various compartments are much more difficult to calculate: The correlation of dioxin levels e.g. between liver, muscle fat, subcutaneous fat and blood could vary considerably.

Therefore, if subcutaneous fat or blood samples are taken to predict levels in meat samples, one would have to consider whether these samples are taken at steady-state or no steady-state (e.g. after eliminating the dioxin source land trying to find out when animals can be slaughtered without condemnation of the meat). Subcutaneous fat is less supplied with blood than other tissues; therefore, longer time might be necessary to reach steady-state.

Community Reference Laboratory
for Dioxins and PCBs in Feed and Food
State Institute for Chemical and Veterinary Analysis of Food, Freiburg, Germany

7.3 Determination of fat

The determination of a "true" fat content is a critical issue and was addressed e.g. at the workshop of the CRL/NRL network in November 2008 (see presentation of Erhard Schulte "Analytical aspects of determination of "true" fat content"). In particular, the determination of fat in blood samples is critical with regard to the low fat content (100 ml blood sample would give around 200 - 300 mg of fat) and with regard to the problems to determine reliably "true" fat content in blood: WHO stopped its exposure studies on contamination of dioxins and dioxin-like PCBs in the middle of the 1990ies when a proficiency test on determination of dioxins, PCBs and fat in human milk failed and no laboratory could be identified as qualified laboratory which met all preset criteria. This was mainly the result of problems to derive a consensus value on fat. Therefore, gravimetric methods would have to be compared with results from enzymatic methods e.g. for determination of triglycerides, cholesterol etc.

The relatively low amount of fat extractable from whole blood or serum could also cause problems regarding the required limits of quantification in particular for animal blood. Comparison of lower- and upper bound levels would reveal such possible problems. The relatively high dioxins levels for the low contaminated beef samples reported in table 1 of Philippe Marchand (Table 11 of this working document) could be the result of reported upper bound levels without reporting the (possibly lower) lower bound levels which then would hint at these analytical problems.

7.4 Correlation of dioxin levels in eggs with levels in meat from hens

As example for the correlation of dioxin levels between different compartments and the observed variability, the correlation of dioxin levels in eggs with levels in meat from hens can be used.

Commission Regulation (EC) No 1881/2006 sets maximum levels for PCDD/Fs in foodstuffs. For hen's eggs and egg products, a maximum level of 3 pg WHO-PCDD/F-TEQ/g fat was set. For meat and meat products from poultry, the maximum level is 2 pg WHO-PCDD/F-TEQ/g fat. Mainly free range eggs from small farms are at risk to exceed the tolerance due to the chicken's dioxin intake from soil. If eggs exceed the tolerance, it is important to know whether also poultry meat is expected to exceed the tolerance. Therefore, the correlation of levels of PCDD/Fs and PCBs in eggs with levels in meat from the same hens was examined to give quick guidance in this question (Rainer Malisch and Christian Wambold, EMV - Feed and Food, p. 231 - 233, Organohalogen Compounds 2005). For this, chickens and eggs with elevated dioxin levels were taken from farms where elevated dioxin levels were found in eggs. All chickens had eggs in their Fallopian tube which allowed to determine an animal-specific correlation.

The levels of PCDD/Fs and PCBs in individual eggs from free-range hens can vary between different animals even on the same farm. To derive data on basis of individual

animals, 6 chickens from 5 different farms with elevated dioxin levels in eggs were chosen. After slaughtering, meat and eggs of the Fallopian tube from the same animals were prepared for analysis.

A comparison of the levels found in meat with levels found in eggs of the Fallopian tube shows that in four samples the dioxin levels in meat are nearly the same (ratio meat : egg between 0.9 and 1.2). In these samples, the ratio for PCB-TEQ is generally slightly higher (ratio meat : egg between 1.2 and 1.4). Two samples had considerably higher ratios between meat and eggs of the Fallopian tube (2.2 respectively 2.5 for WHO-PCDD/F-TEQ; 2.1 respectively 2.0 for WHO-PCB-TEQ).

In addition to the meat samples, in three samples also adipose tissue was analyzed. It was found that the ratios fat : eggs were always slighty higher than the ratios between meat and eggs.

In two cases, eggs were collected from the chickens before slaughtering. Analyses of these samples show a variation of the ratios in comparison to the eggs from the Fallopian tube. This variation hints at a biological variation of the dioxin levels in free range chicken. It could be explained by their presumably wide range of levels of differently contaminated sources on their farm and the resulting variation in daily intake. Only in one case, egg samples collected before slaughtering had higher levels of PCDD/Fs and PCBs than the corresponding chicken's meat. Here, the lowest ratio between meat and eggs was found with 0.61 for WHO-PCDD/F-TEQ.

As a result, chicken's meat is contaminated with about the same level of PCDD/Fs and PCBs or with higher levels as eggs in the Fallopian tube. If egg samples were collected before slaughtering, a biological variation became obvious. With regard to legal consequences one has to take into consideration the fact that the maximum level for chicken's meat is 2 pg WHO-PCDD/F-TEQ/g fat in comparison to 3 pg WHO-PCDD/F-TEQ/g fat for eggs. All results of this study showed that also the tolerance for chicken's meat will be exceeded if the eggs exceed the tolerance for dioxins.

7.5 Correlation between different matrices from humans

Interesting and important aspects can also be derived from comparison of different human matrices.

In general, a good correlation of 2,3,7,8-TCDD levels (on lipid weight basis) between adipose tissue and serum was found (**Figure 4**, Patterson et al, 1988, from Olaf Päpke, personal communication, May 2009) revealing, however, some variability: It would not be possible to predict <u>exactly</u> the serum adipose level from a given serum level (or vice versa).
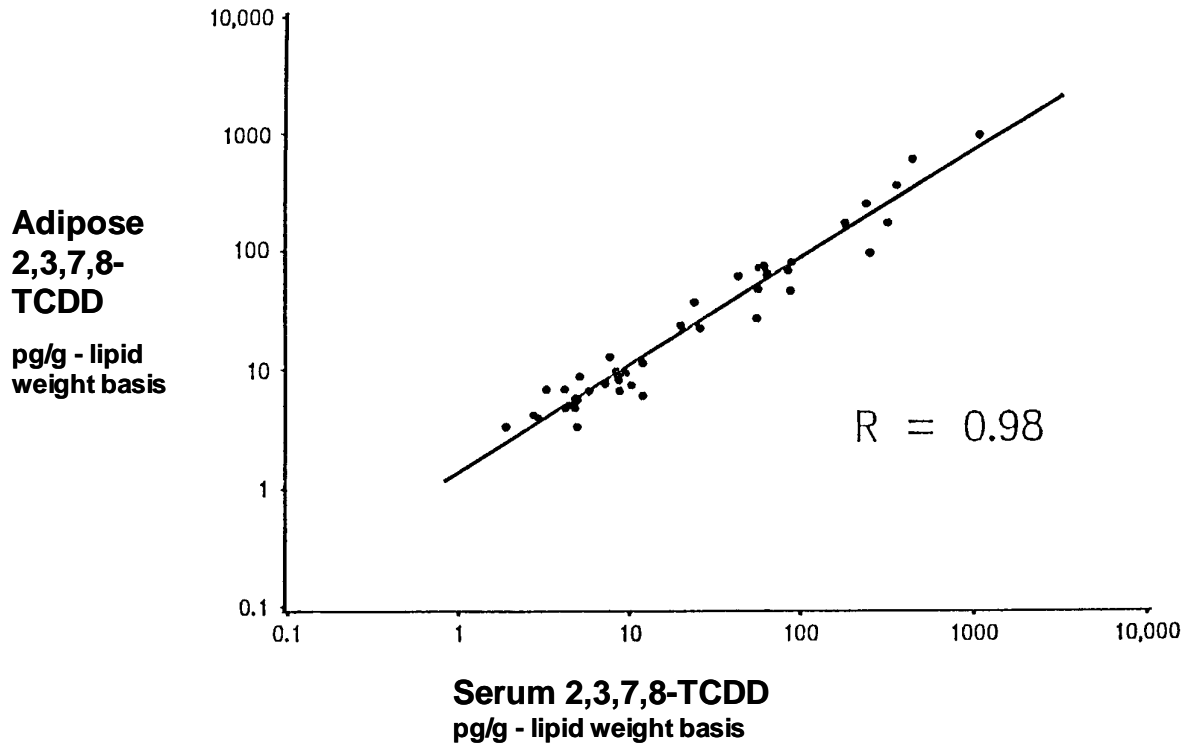
Figure 4: Adipose and serum 2,3,7,8-TCDD-levels (Patterson et al, 1988)

The congener patterns between human milk and human blood differs slightly for lower chlorinated dioxins and furans but significantly for higher chlorinated dioxins and furans (**Figures 5 and 6**, from Olaf Päpke, personal communication, May 2009):
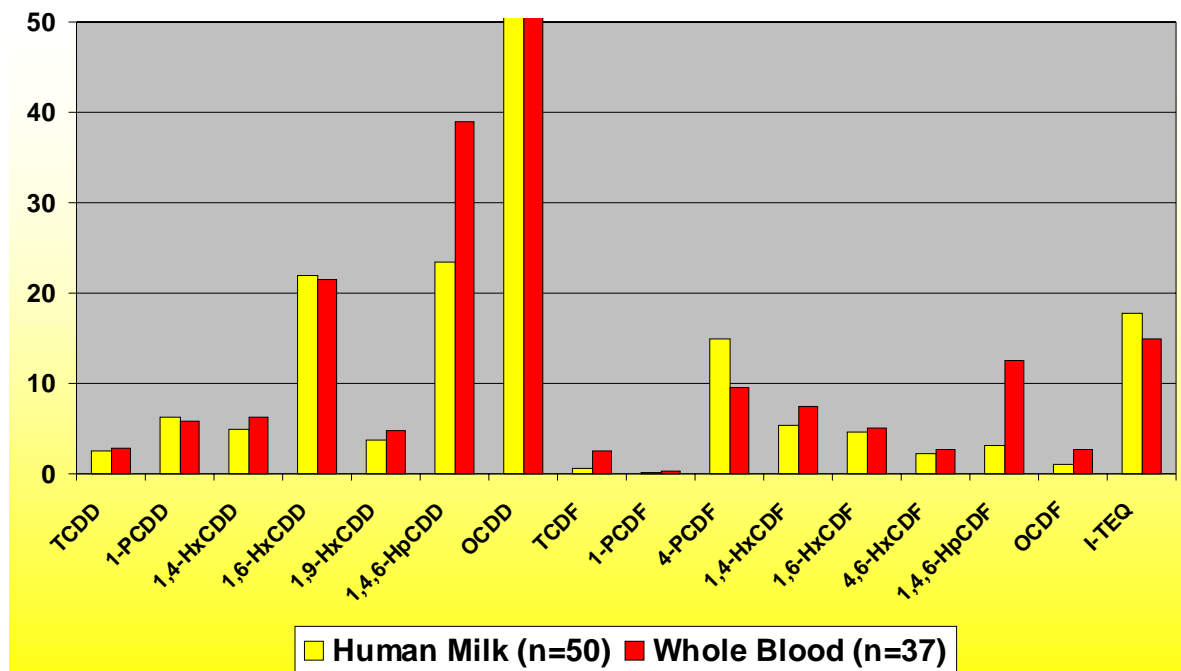

Figure 5: PCDDs / PCDFs in humans - comparison of average values for blood and milk, 1994 (values in pg/g fat) (Olaf Päpke, 1998; personal communication, May 2009)
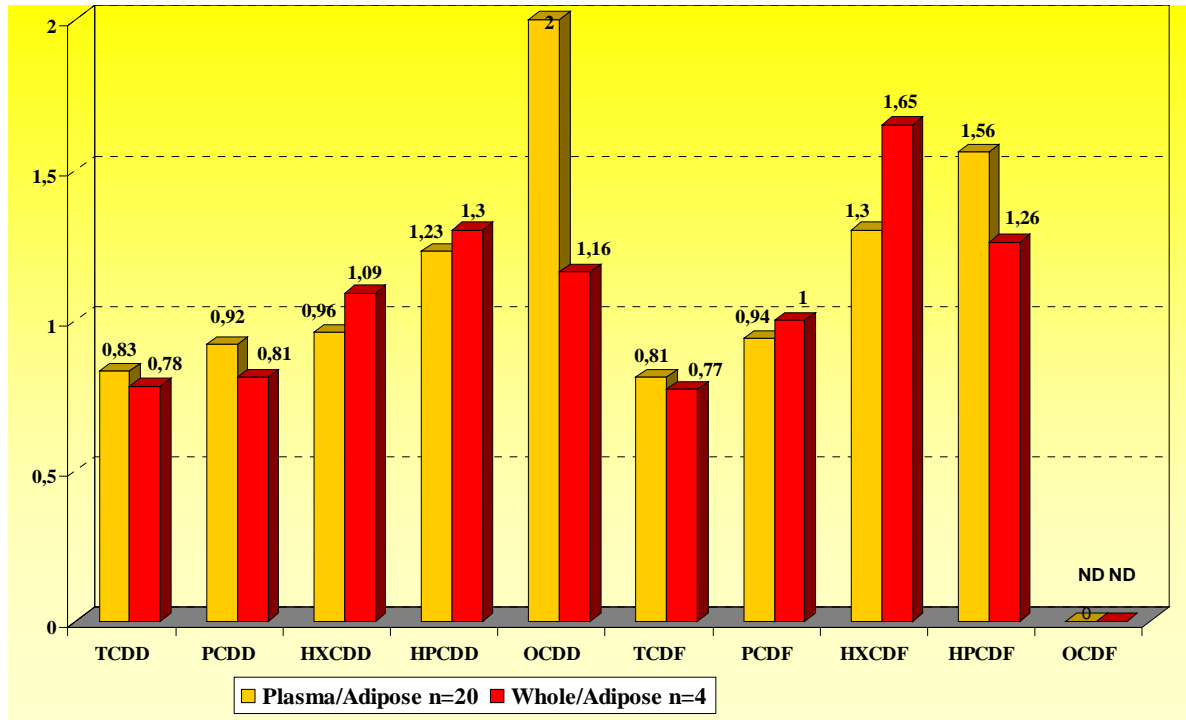
Figure 6: 2,3,7,8-PCDD/F ratios in humans: plasma / adipose and whole blood / adipose - (Schecter, Päpke, Ryan 1991; Olaf Päpke personal communication, May 2009)

Figure 7 demonstrates, that human blood, adipose, liver and kidney have quite comparable dioxin levels on fat basis - however, some variability of the reported levels was observed (Zober and Päpke, 1995; Olaf Päpke personal communication - May 2009).
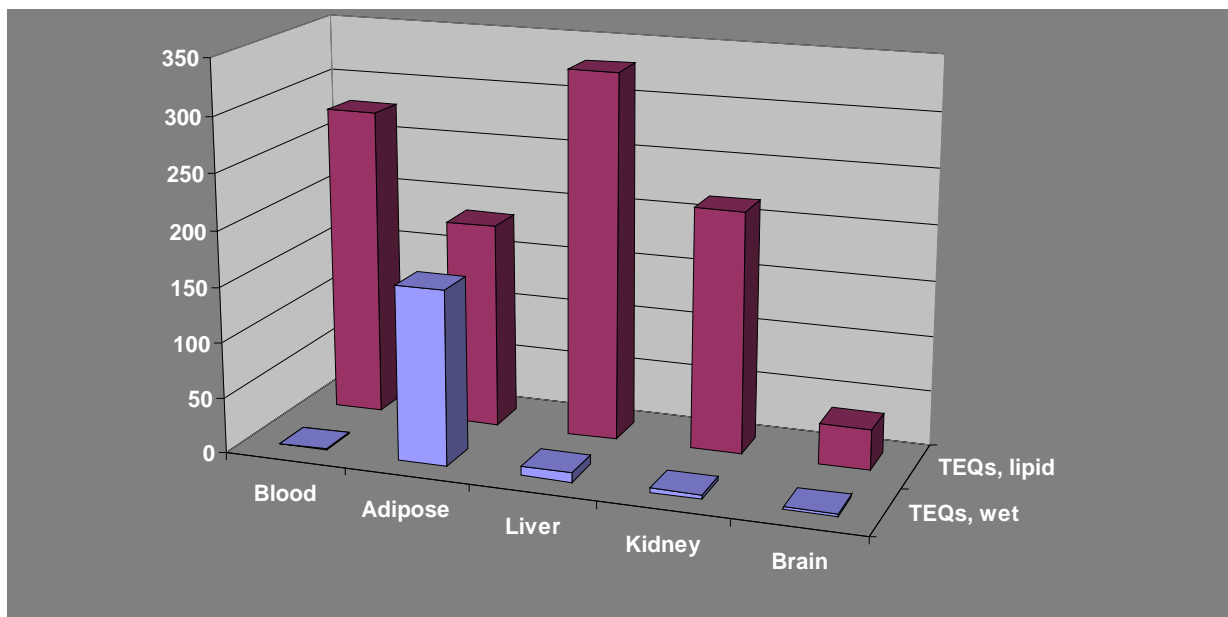

Figure 7: TEQs in various human tissues: comparison of lipid and wet weight based values in pg/g (Zober and Päpke, 1995; Olaf Päpke personal communication, May 2009)

Marchand et al provided the following data related to dioxin and PCB concentrations in various human adipose tissues at low, medium and high contamination levels which confirm their observations in animals (i.e. no significant differences between deep and superficial fat) (**table 12 and figures 8 - 9**):

Sexe	Age (ans)	Origin	% fat	OMS TEQ dioxins (pg/g fat)	OMS TEQ PCB DL (pg/g fat)	Somme PCB indicateurs (ng/g fat)	mean TEQ diox	CV TEQ diox (%)	mean TEQ PCBDL	CV TEQ PCBDL (%)	mean PCB ind	CV PCB ind (%)
woman	53	superficial adipose tissu	47,29	18,73	37,07	569,17	17,09	13,52	31,90	22,92	520,80	13,13
		deep adipose tissu	73,29	15,46	26,73	472,43						
man	34	deep adipose tissu	70,68	8,51	13,15	189,81	8,44	1,07	12,47	7,65	189,88	0,05
		superficial adipose tissu	56,07	8,38	11,80	189,95						
woman	29	superficial adipose tissu	82,53	6,58	6,39	83,37	6,73	3,01	6,00	9,25	75,10	15,57
		deep adipose tissu	76,40	6,87	5,61	66,84						
woman	51	deep adipose tissu	66,87	26,56	85,52	2117,96	26,62	0,33	88,06	4,07	2146,16	1,86
		superficial adipose tissu	78,11	26,68	90,59	2174,36						
woman	49	deep adipose tissu	56,77	13,23	19,39	358,01	12,33	10,34	18,30	8,45	340,08	7,46
		superficial adipose tissu	74,78	11,43	17,20	322,15						
woman	26	deep adipose tissu	76,58	6,44	6,55	81,40	6,43	0,24	6,29	5,94	81,75	0,60
		superficial adipose tissu	75,06	6,42	6,02	82,10						
woman	46	deep adipose tissu	74,17	16,08	40,20	1210,02	16,31	2,01	41,01	2,76	1201,35	1,02
		superficial adipose tissu	68,23	16,54	41,81	1192,68						
woman	40	deep adipose tissu	52,51	10,28	18,30	294,44	10,21	1,01	17,80	4,03	285,81	4,27
		superficial adipose tissu	70,96	10,13	17,29	277,19						
man	29	superficial adipose tissu	55,76	4,36	7,37	96,76	4,58	6,72	6,88	10,09	89,04	12,26
		deep adipose tissu	64,91	4,80	6,39	81,33						

Table 12: Levels of dioxins and PCBs in various human adipose tissues (P. Marchand et al, personal communication, May 2009)

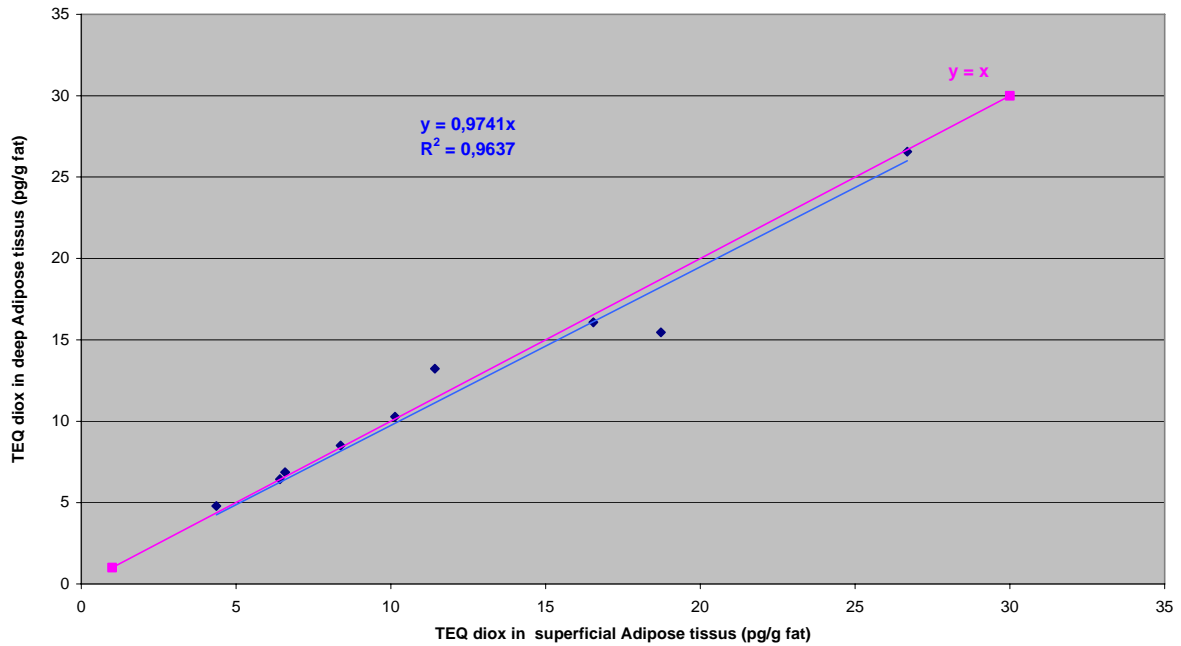**Corrélation between diox TEQ in deep and superficial tissus on human**

Figure 8: Correlation of dioxin TEQ between deep and superficial human tissues (P. Marchand et al, personal communication, May 2009)

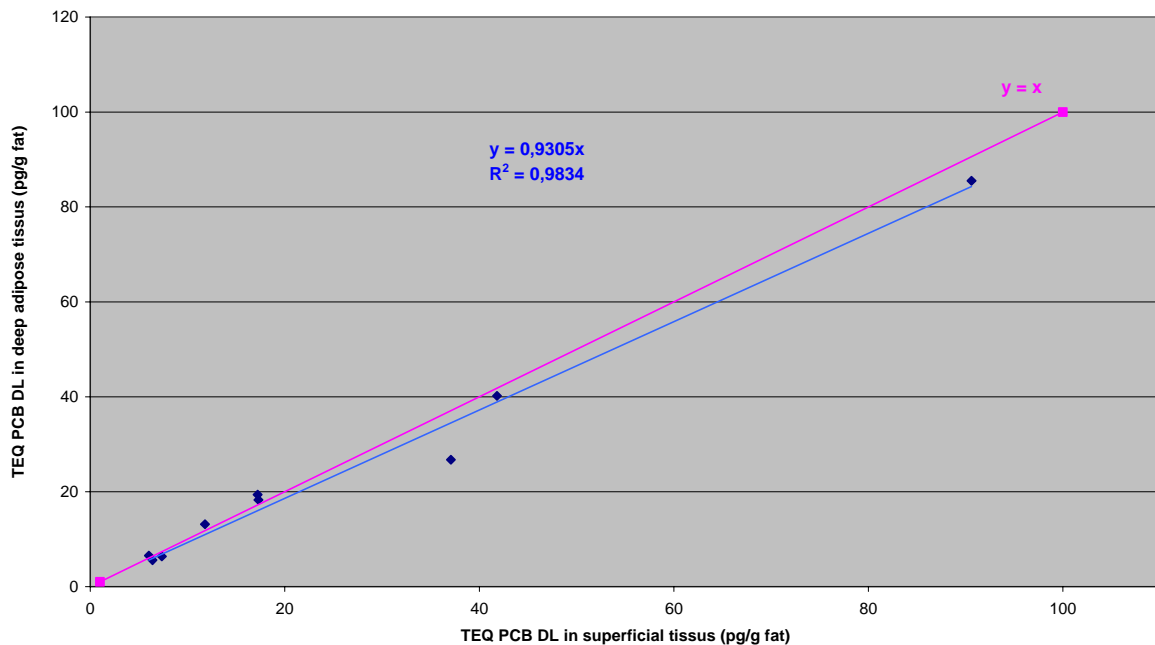**Corrélation between TEQ PCB DL in deep and superficial adipose tissus**

Figure 9: Correlation of PCB-TEQ between deep and superficial human tissues (P. Marchand et al, personal communication, May 2009)

7.6 Correlations of indicator PCB levels between blood, meat and fat

The correlation of indicator PCBs levels between blood and meat was investigated by Johannes Hädrich and Frank Baum (Beurteilung der PCB-Belastungssituation landwirtschaftlicher Nutztiere durch Bestimmung des PCB-Gehaltes im Blutplasma, Archiv für Lebensmittelhygiene 1992, **43**, 73 – 96). The authors derived

- a warning level (PCB levels in plasma below these warning levels would guarantee the compliance of meat samples with maximum levels for meat according to German regulations with 95 % probability);

- a maximum level (PCB levels in plasma exceeding these maximum levels would indicate that the maximum levels for meat according to German regulations would be exceeded with 95 % probability).

These warning and maximum levels were established for the PCB-congeners 138, 153 and 180 (**table 13**) and confirmed in the daily routine of the CVUA (Chemisches und Veterinäruntersuchungsamt) Freiburg.

Beef	Basis	PCB 153	PCB 138	PCB 180
Max Level for meat (Germany, SHMV	mg/kg fresh weight	0.01	0.01	0.008
Warning level	µg/kg blood plasma	0.32	0.33	Not derived
Maximum level	µg/kg blood plasma	1.44	1.12	1.26

Table 13: Warning level and maximum levels for indicator PCB in blood plasma

P. Marchand, A. Vénisseau, A. Brosseaud, C. Gadé-Hildevert, F. Ramdin and B. Le Bizec conducted a study in bovine ("Study of a relationship between PCB concentrations in bovine serum, fat and muscle samples") showing also a good correlation between DL-PCB concentration levels in various compartments (with very close equations compared to those found for dioxins), see excerpt of the paper below. One tested concentration level is at 3 pg/g of fat (no significant differences with higher levels of contamination). In this case the concentration level in muscle is still 20-30% below the one in fat.

Abstract

The purpose of the present study was to analyse four different kinds of bovine matrices (liver, serum, fat and muscle samples) in order to investigate the relationship between the PCB concentrations in those different compartments of the animal. A total of 12 slaughtered castrated males were studied. In all 12 of them, we analysed the PCB level in blood as well as in a pool of muscles and in 4 of them, we made a complete analysis of muscle, fat, liver and blood samples. Our results seem to show no difference statistically significant in terms of PCB levels in all fat tissues and in 3 of the 4 muscle tissues. We demonstrated a correlation between the PCB levels of fat taken by biopsy from behind the ear of the animal and the muscles. We also showed a good correlation between the results for blood and the pool of muscles.

Introduction

Although polychlorinated biphenyls (PCB) have been banned in all industrialised countries for more than 30 years they may still be present in most of the environmental matrices in Northern countries. The consequence is that those compounds may be present at a more or less high level in animals, which could cause concern for human health. The current regulation regarding dioxins and PCB in food is based on maximal tolerable limits in various edible tissues or products, the advantage being that the Authorities have fixed a maximum limit for each kind of matrix. However, one of the drawbacks of this system is that it is difficult to perform an efficient and rapid control on live animals. Indeed, the determination of PCDD/F and PCB levels in muscle clearly implies animal slaughtering. On live animals, however, serum is recognized as a good indicator of exposure and is easy to collect. But since it is not directly consumed or used, no limits have been established for this matrix. Moreover, maximum limits have been fixed in fat and muscles, but no correlation has been demonstrated between all the classes of these matrices. In this context a few questions need to be asked:

- Is there homogeneity of the tissues?

- Is the result of a biopsy predictive of the value we could find in the muscle?

- Is it possible to consider blood as representative of the charge?

Materials and Methods

Samples

Animals were castrated males between 3 and 4 years old naturally grazed with hay contaminated by PCB. Twelve animals were slaughtered and 10 samples were taken on each animal in order to compare the contamination. Different kinds of muscles were taken from the neck, the shoulder, the prime cut of the beef (thick skirt or hanging tender) and finally on the topside (outside flat or bottom round). For the fat, internal fat was taken (peritoneal and perirenal) and external fat corresponding to superficial fat was taken from an easy collection spot (fat from the ear and the neck). A sample of blood and a part of the liver were also collected. The 12 blood samples and 12 muscle pools were analyzed, but only 4 animals were completely studied.

Extraction and clean-up

Blood sample was collected without an anticoagulant, centrifugated and the upper layer corresponding to the serum was withdrawn. The 18 ^{13}C-labelled standards (12 dioxin-like PCB and 7 markers PCB) were added to the serum sample before extraction. After spiking, the sample was diluted with deionised water. Extraction procedure was performed as follows: addition of aqueous saturated ammonium sulphate and ethanol, extraction twice with hexane. The total lipid content of the serum samples was determined using an enzymatic dosage of four classes of lipids on a 50µL aliquot. Before extraction, muscle and liver samples were freeze-dried and the internal standards were added. The extraction was performed using the Accelerated Solvent Extractor (ASE) with a toluene/acetone mixture (70/30, v/v). The solvent was evaporated to dryness, allowing an estimation of the fat content. Fat samples were put in an oven at 105°C overnight and fat was directly taken using a Pasteur pipette. Clean-up and separation processes were carried out using the classic liquid-solid adsorption chromatography with silica, Florisil and CarbopackC/Celite. The solvent used for the elution was hexane. The external standard was added for the recovery calculation ($^{13}C_{12}$-PCB #111 for the 2 fractions of PCB- planar and non planar PCB).

GC/HRMS analysis

GC/HRMS analysis of the 12 dioxin-like and 7 markers PCB was performed as previously described[1]. The congeners were separated by gas chromatography (GC) on a DB-5MS capillary column (30 m × 0.25 mm, 0.25 µm) and determined by high-resolution mass spectrometry (HRMS) on a JMS 700D (Jeol), at a resolution of 10000 in the selected ion-monitoring (SIM) mode using Electronic Impact as ionisation technique. TEQ values were calculated using WHO-TEFs.

Results and Discussion

The PCB concentrations measured in all the samples are presented in Table 1.

animal	matrix	DL-PCB WHO-TEQ (pg/g of fat)	Sum marker PCB (ng/g of fat)	fat content (%)
1900	perirenal fat	2,95 ± 0,60	15.99 ± 3.63	95.09
	peritoneal fat	2,94 ± 0,60	17.29 ± 3.92	94.76
	neck fat	2,96 ± 0,60	15.56 ± 3.53	81.93
	ear fat	2,65 ± 0,54	14.10 ± 3.20	77.96
	neck meat	2,53 ± 0,52	14.46 ± 3.28	6.74
	topside meat	2,47 ± 0,50	14.21 ± 3.22	2.89
	shoulder meat	2,39 ± 0,49	14.00 ± 3.18	6.76
	prime cut meat	2,46 ± 0,50	14.41 ± 3.27	10.30
	pool of muscles	*2,39 ± 0,49*	*13.81 ± 3.13*	*7.61*
	liver	2,91 ± 0,59	39.01 ± 8.85	5.74
1912	perirenal fat	71,32 ± 14,56	193.01 ± 43.79	87.58
	peritoneal fat	70,67 ± 14,43	190.30 ± 43.18	90.38
	neck fat	61,56 ± 12,57	162.66 ± 36.91	70.94
	ear fat	66,70 ± 13,62	176.38 ± 40.02	87.15
	neck meat	55,69 ± 11,37	181.19 ± 41.11	5.56
	topside meat	40,87 ± 08,35	138.17 ± 31.35	1.12
	shoulder meat	51,72 ± 10,56	169.20 ± 38.39	3.05
	prime cut meat	57,77 ± 11,80	187.03 ± 42.44	8.24
	pool of muscles	*49,09 ± 10,03*	*153.89 ± 34.92*	*5.34*
	liver	103,59 ± 21,15	387.50 ± 87.92	5.54
1913	perirenal fat	23,36 ± 4,77	82.38 ± 18.69	91.87
	peritoneal fat	20,31 ± 4,15	71.33 ± 16.19	92.45
	neck fat	21,31 ± 4,35	77.18 ± 17.51	77.58
	ear fat	22,03 ± 4,50	78.80 ± 17.88	88.09
	neck meat	21,48 ± 4,39	90.92 ± 20.63	5.78
	topside meat	16,14 ± 3,30	69.25 ± 15.71	2.14
	shoulder meat	20,68 ± 4,22	86.68 ± 19.67	4.18
	prime cut meat	22,66 ± 4,63	94.59 ± 21.46	5.85
	pool of muscles	*23,64 ± 4,83*	*100.15 ± 22.72*	*3.95*
	liver	36,37 ± 7,43	169.13 ± 38.37	5.61
4633	perirenal fat	15,68 ± 3,20	53.99 ± 12.25	95.66
	peritoneal fat	18,54 ± 3,79	61.55 ± 13.96	93.87
	neck fat	12,38 ± 2,53	40.64 ± 9.22	75.60
	ear fat	15,12 ± 3,09	48.49 ± 11.00	79.72
	neck meat	14,22 ± 2,90	57.97 ± 13.15	3.65
	topside meat	09,95 ± 2,03	40.81 ± 9.26	1.34
	shoulder meat	13,49 ± 2,76	53.25 ± 12.08	3.51
	prime cut meat	15,99 ± 3,27	61.94 ± 14.05	6.20
	pool of muscles	*14,89 ± 3,04*	*57.91 ± 13.14*	*4.39*
	liver	23,91 ± 4,88	85.63 ± 19.43	5.57

Table 1: DL PCB-WHO-TEQ and marker PCB sum in muscle, liver and fat samples.

The values determined are in the same range for all the samples originating from the same animal. We can observe that there is no statistically significant difference between three of the muscles following the ANOVA test. For one of them, if we consider the 3 animals whose concentrations were clearly above the maximum limit, the deviation between the topside and the prime cut meat samples was systematically higher than 28% (respectively 28.8, 29.2 and 37.8). Despite this observation, based on the data obtained, the fat content of the muscles may influence the PCB levels.

correlation between ear fat and other kind of fat

- perirenal fat: $y = 1,0709x - 0,1841$, $R^2 = 0,9999$
- peritoneal fat: $y = 1,0517x + 0,1133$, $R^2 = 0,9941$
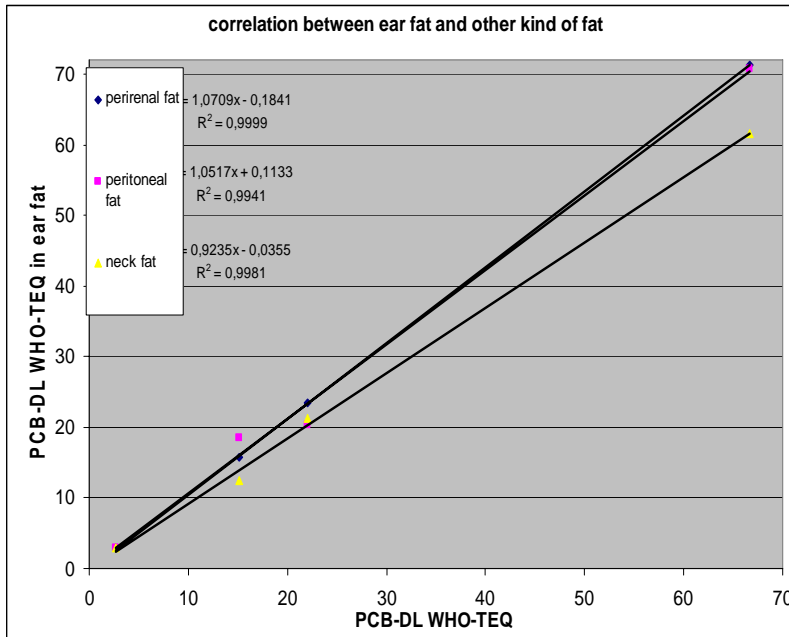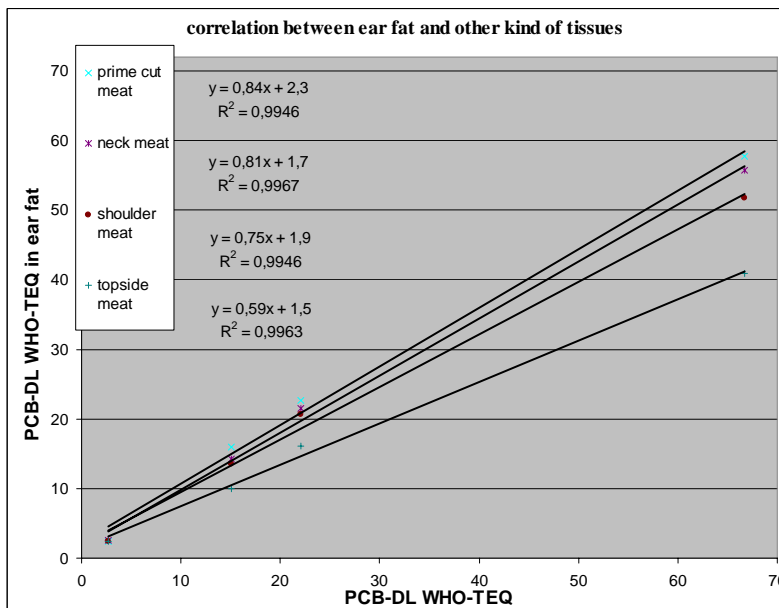- neck fat: $y = 0,9235x - 0,0355$, $R^2 = 0,9981$

Fig 1 shows the relationship between the different kinds of fat. The ear fat was taken as the reference because it is directly accessible by biopsy and the quantity of fat which can be taken at this spot is high.

The correlation found is very good with an excellent correlation factor, which means that this kind of sample could reasonably be allowed for an evaluation of the contamination degree and probably statute on the compliancy of the animal.

Fig 1: correlation between ear fat and the others classes of fat

correlation between ear fat and other kind of tissues

- prime cut meat: $y = 0,84x + 2,3$, $R^2 = 0,9946$
- neck meat: $y = 0,81x + 1,7$, $R^2 = 0,9967$
- shoulder meat: $y = 0,75x + 1,9$, $R^2 = 0,9946$
- topside meat: $y = 0,59x + 1,5$, $R^2 = 0,9963$

The comparison of the results between the different kinds of muscles and the ear fat revealed high correlation factors meaning that ear fat could be a good indicator of the PCB body burden (fig2). However we must not minimize the fact that only the 3 fattiest muscles are close to the value found in the ear fat in terms of concentration.

As an illustration of this comment, the deviation between the mean of the 3 fattiest muscles and the leanest one is in a magnitude of 20%.

Fig 2: relationship between ear fat and the 4 different muscles

It would be interesting to qualify the status of an animal while it is still alive. The fat from the ear taken by biopsy produces an interesting result but this can also be done by blood collection. The correlation between

serum and the pool of the 4 kinds of muscles has been studied. We chose to compare serum and the pool of muscles. Results are presented in Table 2 and Fig 3.

Animal	muscle		blood	
	DL PCBs (pg/g fat)	marker PCBs (ng/g fat)	DL PCBs (pg/g fat)	marker PCBs (ng/g fat)
1900	2.09	16.84	3.14	22.94
1906	2.29	12.69	2.42	18.68
1907	1.99	14.45	3.22	26.01
1910	14.81	43.05	16.64	62.94
1912	59.57	196	68.47	252.66
1913	22.15	100.72	11.61	66.69
1914	16.58	58.35	10.63	41.79
1915	34.61	127.34	33.44	148.93
1920	51.21	225.44	60.51	321.23
4633	12.56	55.51	15.43	80.85
4635	19.76	88.1	16.82	96.22

Table 2: DL PCBWHO-TEQ and marker PCB sum in muscle and blood samples.

Fig 3: Observed correlation between the PCBs concentrations in serum and muscle
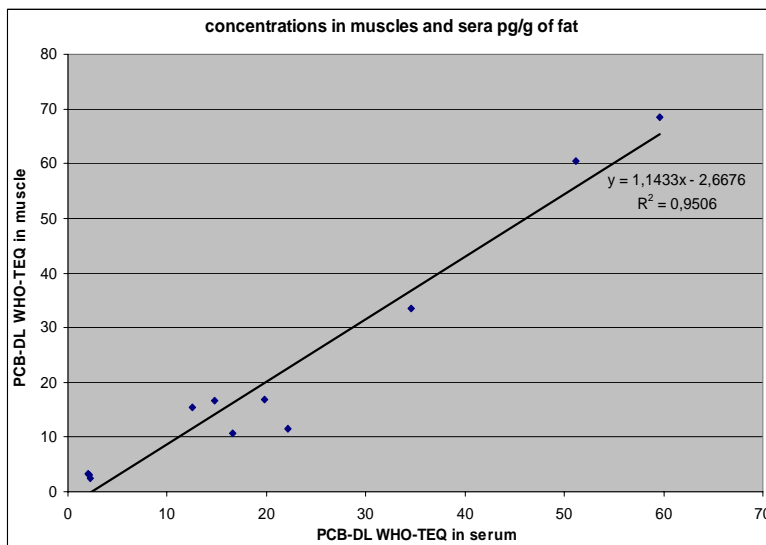
As expected, there is a very good correlation between the serum and the pool of muscles whatever the level of contamination.

Conclusion
According to this study we have observed that all the matrices coming from the same animal are in the same range in terms of concentration. For instance, the results show no difference statistically significant of PCB levels in both fat tissues and muscle tissues. To conclude, it seems relevant to consider that a biopsy of fat behind the ear of bovine can be used to statute on the compliancy of the animal as well as blood sample.

Results obtained by Marchand et al in 2004 on ruminants (goats) which were at the steady-state (experimentation) are shown in **table 14**. In this case again the same factor between muscle and internal fat was observed (i.e. 20-30% less in muscle).

Matrice	N° chèvre	N° ech	OMS-TEQ dioxines (pg/g MG)	OMS-TEQ PCB DL (pg/g MG)	Somme des PCB indicateurs (ng/g MG)
Sérum	1	4.283-16	8.35	0.87	8.37
	2	4.283-17	2.15	0.93	5.50
	3	4.283-18	4.09	3.90	13.37
Muscle	1	4.283-7	2.69	1.06	6.41
	2	4.283-8	3.36	1.11	6.52
	3	4.283-9	2.84	1.21	7.12
liver	1	4.283-19	52.04	5.10	29.95
	2	4.283-20	103.14	11.50	35.40
	3	4.283-21	91.66	12.19	29.58
kidney	1	4.283-13	6.02	0.99	5.74
	2	4.283-14	3.50	0.66	3.04
	3	4.283-15	3.16	0.88	4.51
digestive fat	1	4.283-4	3.39	1.15	7.32
	2	4.283-5	5.13	2.01	9.63
	3	4.283-6	2.83	1.41	6.65
Perirenal fat	1	4.283-10	3.52	1.35	7.02
	2	4.283-11	4.83	1.87	9.20
	3	4.283-12	2.92	1.34	6.31

Table 14: correlation of dioxins, dioxin-like PCBs and indicator PCBs between different tissues of goats (P. Marchand et al, personal communication, May 2009

In all these studies performed by Marchand et al, and either for dioxins or PCB, the concentration found in the muscle is 20-30% below the concentration found in fat. In all these cases (human or ruminants) there is a very good correlation between internal and superficial fat. Last but not least, it should be mentioned that in all these cases the method used to extract the fat was exactly the same either in muscles or in fat (based on ASE with a mixture of toluene and acetone).

7.7 Discussion and Conclusions

Use of the correlation of dioxin levels between different biological samples of the same animal is an important tool to take samples before slaughtering to predict compliance of meat of the animal. For cows, milk could easily be analysed. For cattle or pigs, blood or fat obtained from a biopsy are alternatives. Marchand et al. presented results of a valuable study showing the correlation for dioxin levels (calculated on fat basis) between muscle, blood, sub-caudal fat and peripheric kidney (= perirenal) fat. These data show that samples with clearly elevated dioxin levels (e.g. above 5 pg WHO-PCDD/F-TEQ/g fat) in blood or fat samples obtained from biopsy would result in meat samples which don't comply with existing regulations. There is a lack of data for samples around the maximum level of 3 pg WHO-PCDD/F-TEQ/g fat for bovine meat. With regard to the variability of correlation factors derived from different biological samples, a regression line with prediction intervals would be helpful to derive conclusions also for samples which would guarantee with a required probability (e.g. 95 %) that samples are below the legal limit for meat. It is expected that animals with dioxin levels with some variation around the maximum level (on fat basis) will cause difficulties when levels in meat have to be predicted from blood or biopsy samples. More data would be helpful to answer questions regarding the prediction intervals and in this way to evaluate the time for feeding with uncontaminated feedingstuff before an animal can be slaughtered without fear of condemnation of the meat.