An Executive Summary

Editors' Series
GC×GC-TOFMS in Food and Feed Control: Going Beyond Dioxin Measurements

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Abstract

The measurement of dioxins and dioxin-like compounds in food and feed (ingredients) is an important task within the EU food safety policy. It has been well documented and described in detail in official EU Regulation documents for the last 20 years. Such measurements require the best available gas chromatography–mass spectrometry (GC–MS) technology in terms of sensitivity and specificity. Both gold standard magnetic sector MS and triple-quadrupole MS analyzers, performing in isotope dilution (ID) mode, are used as confirmatory tools for precise and accurate measurements under the EU legislation. The required sensitivity is achieved by performing in selected ion monitoring (SIM) or single- or multiple-reaction monitoring (S/MRM), limiting the measurements to the specific target analytes for which EU maximum levels were established. Comprehensive two-dimensional GC coupled with time-of-flight MS (GC×GC-TOFMS), acquiring all masses within a defined mass range, has been used for dioxin measurement in the past, but was suffering from limited sensitivity at the high femtogram level, despite the zone compression effect of the modulator. In this study, we will discuss the use of a new generation of GC×GC-TOFMS systems for targeted and non-targeted measurements of dioxins in food and feed samples.

Chickengate

About 20 years ago, Belgium had an incident known as "Chickengate," where large numbers of chickens and pigs were dying of dioxin poisoning. It took about six months for the food safety authorities to figure out what was going on, but in that time, the people of Belgian and the neighboring countries that imported the livestock were potentially exposed to high dioxin levels. Eventually when the problem was understood, about seven million chickens and 50,000 pigs were slaughtered at a cost of almost two billion euros to the Belgian government.

The source of the problem was the incorporation of polychlorinated biphenyl (PCB)-contaminated mineral oil into fat dedicated to animal feed production. Overall, approximately 80 tons of fat (500 tons of feed) was contaminated with 50 kg of PCBs and 1 g of polychlorinated dibenzofurans (PCDFs). This translated into many of the chickens being at a toxic equivalent quantity (TEQ) level of 950 µg (micrograms) of dioxin per gram of fat. To put that in perspective, the legal level of dioxin in foodstuff is 1–5 µg TEQ, depending on the product. So as a result, it gained a great deal of attention in Belgium when so many of the chickens had to be destroyed.

Following this investigation, stricter food and feed regulations in terms of dioxin and related chlorinated hydrocarbons compounds were implemented. They included controls of seven polychlorinated dibenzodioxins (PCDDs; 2, 3, 7, 8-substituted tetra- to octa-chlorinated compounds), 10 PCDFs (2, 3, 7, 8-substituted tetra- to octa- Cl), four NO (non ortho)-PCBs, and eight MO (mono ortho)-PCBs.

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Toxic Equivalent Factor Values

Table 1 shows the toxicity of the major chlorinated hydrocarbon compounds regulated by the WHO (World Health Organization). PCDDs are the most toxic, with a toxic equivalent factor (TEF) of 1. Then, the toxicity decreases with the PCDFs and the NO-PCBs; the MO-PCBs are the least toxic. At this moment, the commercial fishing industry is driving this issue and pushing for lower levels but we all must be realistic about what's achievable. If we are going to move in the direction of continuous monitoring on a daily basis, then we must have significant analytical capabilities.

Screening

For this reason, regular screening known as the Rapid Alert System for Food and Feed (RASFF) system has been adopted by the EURL/NRL/OFL European network of laboratories. This alert system ensures that as soon as a high level is measured somewhere in Europe, the information is relayed to all participating laboratories so that action can be taken very quickly, which clearly was not the case during the "Chickengate" crisis. This process works on a maximum action level strategy where the maximum level is the level above where you cannot eat or commercialize the food. So, if something is above the action level, we check to see why the level is high and what should be done to get it lower. Those familiar with dioxin measurement know those levels are very low (i.e., in the order of low-picogram or high femtogram concentration level), so you are very often facing samples that are not-detectable—even using a highly sensitive magnetic sector instrument. When this happens, you can either report the congener non-detects as zero or as below the limit of detection (LOD) or limit of quantification (LOQ). If the LOQ value is used for the non-detects, it is known as the *upper-bound approach*, which is considered an over-estimation of the value of the concentration. Meanwhile, the *lower-bound approach* is considered an under-estimation of the value. So, these estimation values must be reported so that the scientific community can decide with all the information available whether the sample is suspicious or not.

Table 1: WHO Toxicity Equivalent Factor (TEF) values for the major chlorinated hydrocarbon compounds.

Congener	TEF value	Congener	TEF value
Dibenzo-p-dioxins ('PCDDs')		**'Dioxin-like' PCBs Non-ortho PCBs + Mono-ortho PCBs**	
2,3,7,8-TCDD	1	*Non-ortho PCBs*	
1,2,3,7,8-PeCDD	1	PCB 77	0,0001
1,2,3,4,7,8-HxCDD	0,1	PCB 81	0,0003
1,2,3,6,7,8-HxCDD	0,1	PCB 126	0,1
1,2,3,7,8,9-HxCDD	0,1	PCB 169	0,03
1,2,3,4,6,7,8-HpCDD	0,01		
OCDD	0,0003		
Dibenzofurans ('PCDFs')		*Mono-ortho PCBs*	
2,3,7,8-TCDF	0,1	PCB 105	0,00003
1,2,3,7,8-PeCDF	0,03	PCB 114	0,00003
2,3,4,7,8-PeCDF	0,3	PCB 118	0,00003
1,2,3,4,7,8-HxCDF	0,1	PCB 123	0,00003
1,2,3,6,7,8-HxCDF	0,1	PCB 156	0,00003
1,2,3,7,8,9-HxCDF	0,1	PCB 157	0,00003
2,3,4,6,7,8-HxCDF	0,1	PCB 167	0,00003
1,2,3,4,6,7,8-HpCDF	0,01	PCB 189	0,00003
1,2,3,4,7,8,9-HpCDF	0,01		
OCDF	0,0003		

Source: WHO, Rev. 2005.

The LOQ will, therefore, affect the upper-bound value, which is why the use of specific instrumentation could have a significant bearing on the value measured. This is exemplified in **Table 2**, which shows the maximum contaminant levels of dioxin in foodstuff per the EU legislation document Commission Regulation (EC) No 1881/2006 (Rev 2018). For poultry, for example, the maximum level in Europe is 1.75 picograms/gram of fat, for the total sum of dioxin and furan (PCDD). However, if you add the dioxin-like PCBs, the TEQ increases to 3.0 picograms/gram of fat. For the other nondioxin-like PCBs, known as the seven-indicator PCBs shown in the third column, the value goes up over three orders of magnitude to 40 nanograms (40,000 picograms)/gram of fat because these compounds are significantly less toxic.

Instrumental Techniques

It is worth pointing out that 20 years ago, high-resolution magnetic sector mass spectrometry was the only tool recognized as the confirmatory method to perform dioxin measurements when a suspected sample had

to be positively confirmed. Then five years ago, triple-quadruple in MS/MS mode was recognized as an acceptable alternative to sector instruments for performing confirmatory analysis at these levels. Therefore, samples below the cut-off value are acceptable because they are not above the maximum value of the biological equivalent quantity (BEQ). Samples above the BEQ must be confirmed by either magnetic sector GC–HRMS or triple-quadrupole MS/MS analysis. In addition, samples that are in between the maximum BEQ value and the cut-off value by a certain percentage can be double-checked to confirm that the bioassay is not giving any false negatives. As a result, many thousands of samples can be screened and double-checked using GC–HRMS or GC–MS/MS systems. This information is then posted on the EU portal to ensure that every country in Europe has access to the latest data of dioxin measurement in food and feed as soon as it becomes available. The benefit of this system is that it keeps pressure on the producers to get dioxin levels even lower.

Table 2: European TEQ values for dioxin compounds in animal fats.

- ✓ Maximum and Action levels
- ✓ Lower / Medium / Upper-bound approach
- ✓ Upper femtogram (10^{-15} g) range

Section 5: Dioxins and PCBs (31)

	Foodstuffs	Maximum levels		
		Sum of dioxins (WHO-PCDD/F-TEQ) (32)	Sum of dioxins and dioxin-like PCBS (WHO-PCDD/F-PCB-TEQ) (32)	Sum of PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180 (ICES – 6) (32)
5.1	Meat and meat products (excluding edible offal) of the following animals (6):			
	— bovine animals and sheep	2,5 pg/g fat (33)	4,0 pg/g fat (33)	40 ng/g fat (33)
	— poultry	1,75 pg/g fat (33)	3,0 pg/g fat (33)	40 ng/g fat (33)
	— pigs	1,0 pg/g fat (33)	1,25 pg/g fat (33)	40 ng/g fat (33)

Source: Commission Regulation (EC) No 1881/2006 (Rev 2018).

The inherent problem with high-resolution magnetic sector MS instrument operating in single ion monitoring (SIM) mode or triple-quadrupole MS system using MRM is that analysts will only see the ions they are looking for. However, using TOFMS, which offers the capability of monitoring all the masses in a defined range at the same time, you get a full permanent scan of all the ions in the sample. However, to take advantage of that capability, you also must couple the TOF instrument with an efficient gas chromatographic approach such as two-dimensional gas chromatography (GCxGC) because you could possibly be looking at hundreds of dioxin and furan congeners to be separated.

This, therefore, became our incentive to couple GCxGC with TOFMS to be used as a realistic and practical alternative to GC–ID–HRMS for routine ultra-trace measurement of PCDD/F congeners in challenging food matrices. This is exemplified in **Figure 1**, which shows that even back in 2005, we were trying to compare GCxGC TOFMS with ID–HRMS and triple-quadruple MS analysis, when in fact the triple-quad technique was not yet accepted by the EU legislation. It can be seen that these pre-techniques were all performing very similarly in terms of profile of the congener and the concentration of the separated congener bases.

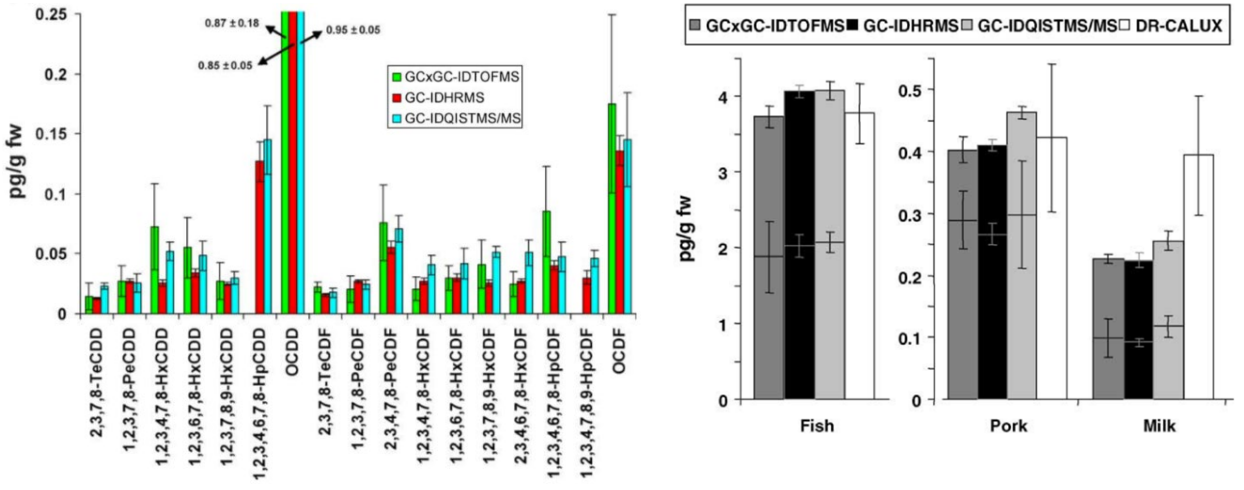
Multidimensional GC-TOFMS

The conclusion from our work in 2005 was that we should take another look at TOFMS analysis because the technique had come a long way with regard to sensitivity. The new generation of TOFMS analyzers not only offer significantly higher sensitivity, but they also have the capability of faster data acquisition to process the large amounts of mass spectral data being generated. For that reason, we decided to look at a new state-of-the-art GCxGC-TOFMS (Pegasus® BT 4D, LECO Corp., St Joseph, MI), which is a small bench-top instrument. We carried out an initial evaluation by running some chlorinated and brominated dioxin standards, together with a couple of real-world samples to see what that system could or could not do. The instrument is shown in **Figure 2**.

The Pegasus® BT 4D GCxGC-TOFMS

The mass range used on the instrument was 40–550 amu, basically a range that covers the dioxins. We acquired data at a frequency of 200 Hz, which might sound fast, but it should be emphasized that this is two-dimensional GC, so the peak width can be as narrow as 50–100 ms. This means your mass spectra acquisition must be rapid enough to characterize the very narrow peaks. In absolute terms, it would be considered a low-resolution mass spectrometer, but probably medium resolution in GC separation terms because it is approximately 50–150 ppm in terms of mass accuracy. The typical MS and chromatographic separation

Figure 1: Comparison of GCxGC–TOFMS with ID–HRMS and triple-quadruple MS with regard to profile of the congener and the concentration of the separated congener bases.



Estimated percent distribution of the cost of the various stages of the measurement methods in the case of feed samples

	GC–ID-HRMS	GC × GC–ID-TOF-MS	GC–ID-QIST-MS/MS	DR-CALUX[a]
Scientist employment	23	35	35	36
Extraction	11	8	11	7
Clean-up	28	27	33	29
Measurement	38	30	21	8
Licensing and royalties	–	–	–	20
Cost per sample (relative)	+++++	+++++	++++	+++

[a] Cost based on duplicate measurements.

operating parameters for dioxins using this technique are shown in **Figure 3**.

It can be seen that the GC phases were 5% in the first dimension and 50% in the second dimension. Of course, the second dimension is much shorter than the first one because it is carried out in fast GC mode. And if you look at the typical TOF chromatographic peaks in the time domain, it can be seen (circled in red) on the top from left to right, the dispersion of the different tetra-, penta-, hexa-, and hepta-molecules, whereas on the bottom, you see the octa-molecule. This is known as the wrap-around effect because the retention times of the two octa-molecule peaks circled in red on the bottom were longer than the second-dimension length. So as a result, they were wrapped around, and just appeared in the next cycle. The peaks might be slightly broader because they stay longer in the column, but as long as they do not co-elute with anything else, this is perfectly acceptable.

Performance

Let's take a closer look at the performance of the instrument. We generated a few calibration curves between 50 femtograms and 50 picograms, which is basically the range where we need to measure these compounds. For example, the hepta- and octa-chlorinated compounds are typically at the picogram level, whereas the tetra- and penta- compounds are much lower at the femtogram level. **Figure 4** is a typical MS spectral fingerprint from 315 amu to 340 amu, which shows the M, M+2, and M+4 ions of the native C^{12} as well as the C^{13} label at one picogram, which was a mixture we made to test the performance of the instrument.

Based on this data, the mass accuracy was approximately 60 ppm. In addition, one other performance criteria when carrying out dioxin measurement is to check the isotope

Figure 2: The Pegasus® BT 4D GCxGC-TOFMS from LECO Corp.

- ✓ Routine sample prep (PLE, Power-Prep™)
- ✓ Samples:
  - Egg from SVFI Slovakia / EU-RL Freiburg
  - Bentonite
- ✓ GCxGC-TOFMS LECO Pegasus® BT 4D

ratio because we know the typical ratio between M, M+2, and M+4, for example, should be less than a 5% of deviation from what is expected. The calibration plots of two of the congeners of penta- and hexa-dioxin, together with tetra- and octa-furan are exemplified in **Figure 5**, which convinced us that the first round of testing looked to be very promising.

Table 3 shows a list of PCBs, dioxins, and furans with their most abundant isotope. For each of them, we have a tolerance of 0.3 Daltons in terms of mass, where we have both the qualifying and the quantifying ions that are used to measure the isotope ratio. Therefore, a great deal of QA/QC checking is required to ensure that when we see a peak, we can say categorically that this is the peak of interest. The table also shows the start time and end time of the scan together with the minimum integrated area/height required to confirm a peak, which was confirmation for us that congener pattern is critically important for the identification of potential contamination using this technique.

Figure 3: Typical mass spectrometry and chromatographic separation operating conditions of the LECO Pegasus GCxGC-TOFMS for dioxins.

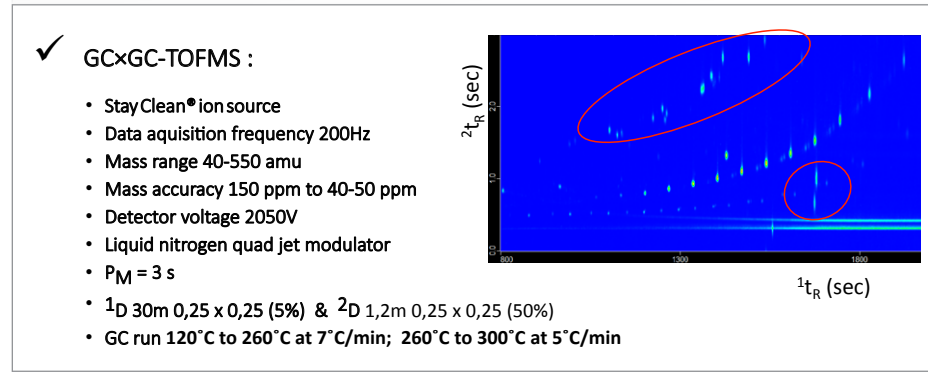
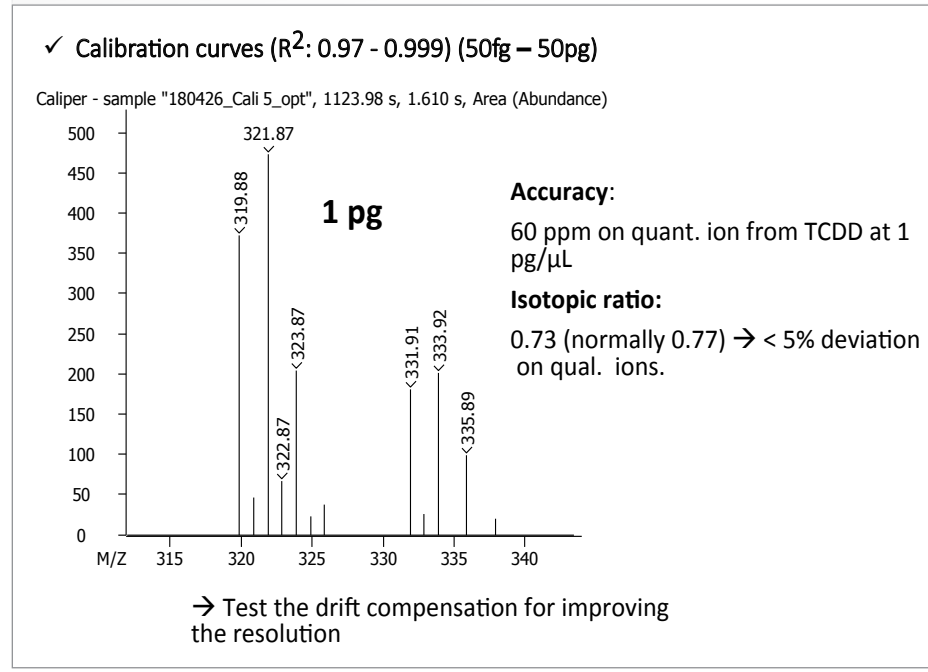


Figure 4: A typical MS spectral fingerprint of TCDD at 1 picogram.



An example of the type of signal that we can expect for TCDD by cranking up the detector voltage from its default value is shown in **Figure 6**. We did this because we wanted to see what was possible in terms of detection capability. The result was that by increasing the voltage by 200 volts, we could get a factor of 10x enhancement in sensitivity. This is exemplified in the top scan of **Figure 6** by comparing the green trace (calibration point 1) to the orange trace (calibration point 3). Then by increasing the detector offset, the integrated peak for calibration point 1 is almost the same as calibration plot 3.

The detector offset voltage signal enhancement capability is further demonstrated in **Figure 7**, with a scan of 10 femtograms of TCDD on the left. Just to put this in perspective, most dioxin labs have a TCDD daily sensitivity check somewhere between 50 and 80 femtograms. And on the right is 100 atograms of tetrabromodibenzodioxin (TBDD). The reason we use TBDD instead of TCDD is because when we were initially injecting the TCDD standards, it was contaminated with some other compounds. When you are trying to measure at sub-femtogram, or high-atogram levels, you must have very pure solutions. And because we don't do bromination on a routine basis in our laboratory, the brominated solutions were far cleaner in terms of contamination. It is also worth emphasizing that the isotopic pattern of the C^{12} peaks and C^{13} peaks on the left and right, respectively, of each mass spectral display were still in very good agreement with the expected isotope ratios, even at such low concentrations.

Real World Samples

Bentonite. To verify our studies, we decided to investigate a couple of real samples that reflected areas of concern in food and animal feed. First, we took a look at bentonite, a natural clay that is one of many ingredients in animal

Figure 5: Calibration plots of two of the congeners of penta- and hexa-dioxin, together with tetra- and octa-furan.

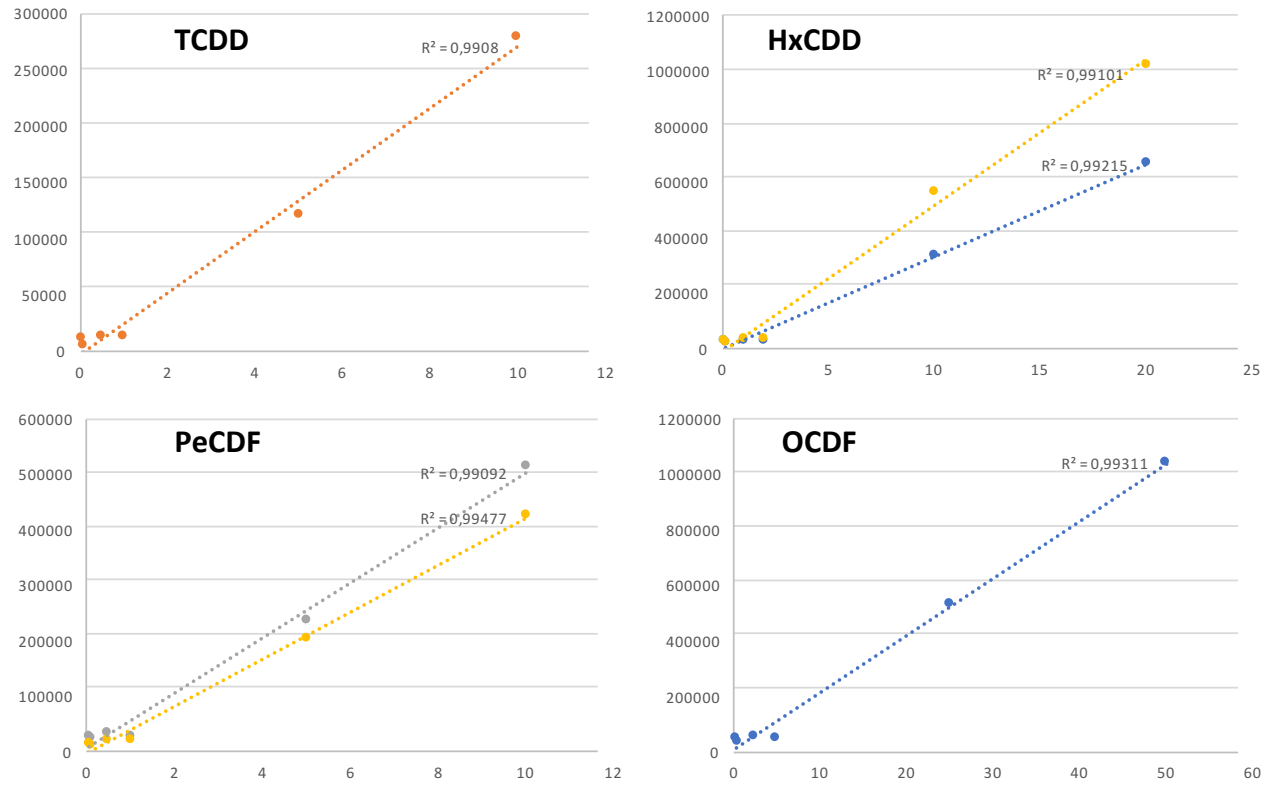


Table 3: Figures of merit for this technique, which confirms that congener pattern is critically important for the identification of potential contamination of dioxin compounds.

Analyte	Formula	Most Abundant Isotope	Tolerance	Units	Start Time	End Time	Min Area	Min Height
TCB-1	C12H6Cl4	291.92	0.3	Da	988.988 s	994.988 s	100	25
TCB-2	C12H6Cl4	291.92	0.3	Da	1003.99 s	1009.99 s	100	25
TCDF	C12H4Cl4O	305.9	0.3	Da	1099.98 s	1105.98 s	100	25
TCDD	C12H4O2Cl4	321.89	0.3	Da	1120.98 s	1126.98 s	100	25
PeCB	C12H5Cl5	325.88	0.3	Da	1135.98 s	1141.98 s	100	25
PeCDF-1	C12H3Cl5O	339.86	0.3	Da	1219.97 s	1225.97 s	100	25
PeCDF-2	C12H3Cl5O	339.86	0.3	Da	1246.97 s	1252.97 s	100	25
HxCB	C12H4Cl6	359.84	0.3	Da	1255.97 s	1261.97 s	100	25
PeCDD	C12H3Cl5O2	355.85	0.3	Da	1258.97 s	1264.97 s	100	25
HxCDF-1	C12H2Cl6O	373.82	0.3	Da	1354.96 s	1360.96 s	100	25
HxCDF-2	C12H2Cl6O	373.82	0.3	Da	1360.96 s	1366.96 s	100	25
HxCDF-3	C12H2Cl6O	373.82	0.3	Da	1384.96 s	1390.96 s	100	25
HxCDD-3	C12H2Cl6O2	389.82	0.3	Da	1387.96 s	1393.96 s	100	25
HxCDD-2	C12H2Cl6O2	389.82	0.3	Da	1393.96 s	1399.96 s	100	25
HxCDD-1	C12H2Cl6O2	389.82	0.3	Da	1405.96 s	1411.96 s	100	25
HxCDF-4	C12H2Cl6O	373.82	0.3	Da	1414.96 s	1420.96 s	100	25
HpCDF-1	C12HCl7O	407.78	0.3	Da	1486.96 s	1492.96 s	100	25
HpCDD	C12H1Cl7O2	423.78	0.3	Da	1531.95 s	1537.95 s	100	25
HpCDF-2	C12HCl7O	407.78	0.3	Da	1552.95 s	1558.95 s	100	25
OCDD	C12Cl8O2	459.73	0.3	Da	1669.94 s	1675.94 s	100	25
OCDF	C12Cl8O	443.74	0.3	Da	1675.94 s	1681.94 s	100	25

feed. We took one sample that was extremely low in chlorinated dioxin levels and injected it into the instrument. Unfortunately, we were unable to see the peaks, which surprised us because the sensitivity of the calibration curve suggested that we should have at least seen some of them. However, we were doing a full mass scan on the TOF instrument and comparing it with SIM on the high-resolution, magnetic sector instrument. So, we were probably being a little ambitious in trying to use the same method. We suspected it might be some kind of matrix suppression, so we decided to modify the sample preparation side to reduce some of the interferences that were potentially affecting the TOF signals. This research is in its early stages, which we hope to present at a later date.

Chicken eggs. Our next project was a very interesting chicken egg sample coming from Slovakia that had been

double checked by many different EU reference labs. With these kinds of samples, the procedure is to first screen with a bioassay method called CALUX (Chemical Activated LUciferase gene eXpression), and then follow up with MS analysis if it is a suspected positive sample. The CALUX result (analyses performed by the State Veterinary and Food Institute in Slovakia and further confirmed by Chemical and the Veterinary Inspection Office in Germany) was around 60 picograms BEQ, which is the biological equivalent of lipid weight. However, the permitted EU levels for the sum of dioxins were 3 picograms and 5 picograms for the sum of dioxins and DL-PCBs. In other words, the EU legislation was saying a maximum of 5 picograms, while the CALUX on this sample was measured at 60 picograms. As you can imagine, this triggered a great deal of discussion because the results by many laboratories using high-resolution mass spectrometry was giving <0.6 picograms TEQ per gram of lipid weight. So, the general consensus was that the CALUX result was suspected as being incorrect. However, as **Table 4** shows, the sample was analyzed by many different types of laboratories using CALUX technology, which all gave results around 60 picograms BEQ. In other words, the biological toxicity of the sample was in the order of 60 picograms BEQ per gram of fat while the MS results were all below 0.6 picograms.

So, this was an area we were very keen to get a better understanding of the problem. If the CALUX result tells you there is high biological activity, you should obviously be very careful about eating such a food product. On the other hand, the MS result was telling us that the level was below 0.6 picograms and therefore was safe to eat. So, if it's not a chlorinated compound, there must be something else that will bind onto the aryl hydrocarbon receptor (AhR). The usual suspects are brominated compounds, AhR-brominated compounds, or mixed-chloro

Figure 6: Increasing the detector offset voltage of 200 volts can enhance sensitivity by 10-fold.

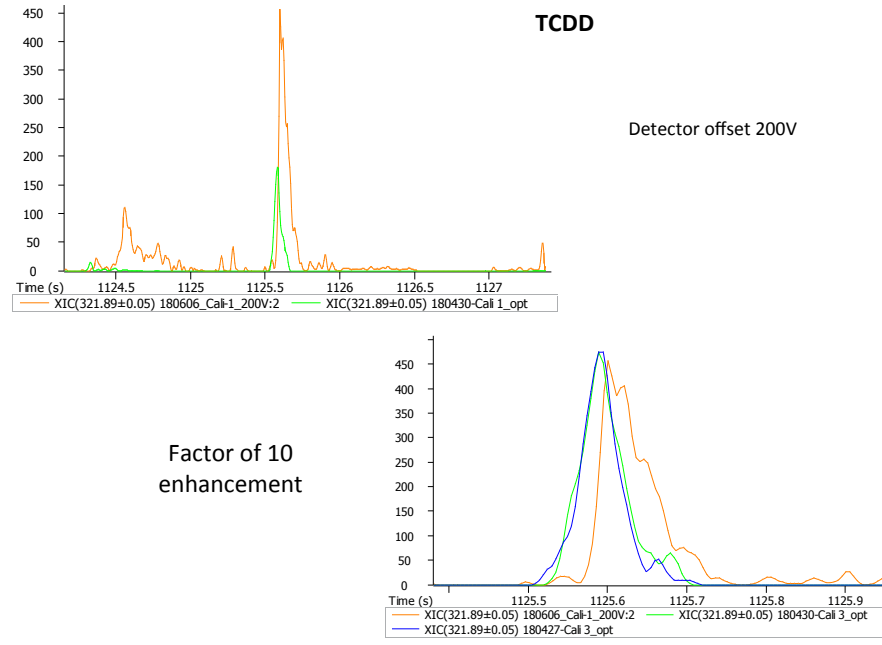


Figure 7: The detector offset voltage signal enhancement capability is further demonstrated with a scan of 10 femtogram of TCDD on the left, 100 atograms of TBDD on the right.

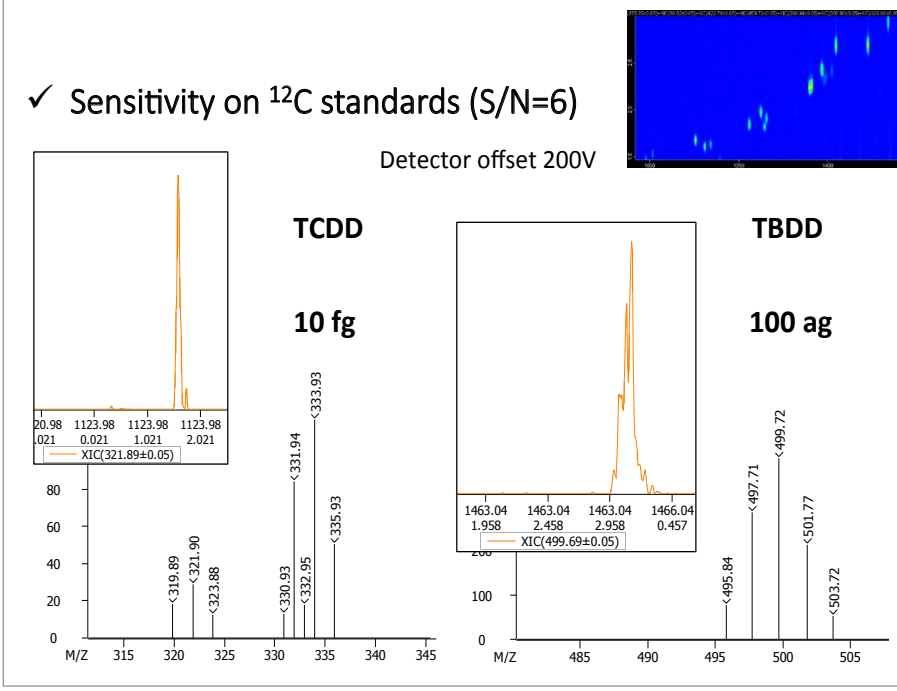


Table 4: Comparison of results between CALUX and GC-HRMS for dioxin related compounds in a chicken egg from Slovakia.

✓ Results confirmed from different laboratories…

Dried Egg Sample - Results			
DR-CALUX (NRL SK)	sum-BEQ	72	pg/g fat
DR-CALUX (BDS)	sum-BEQ	67	pg/g fat
DR-CALUX (EURL)	sum-BEQ	71	pg/g fat
XDS-CALUX (EURL)	PCDD/F-BEQ	31	pg/g fat
XDS-CALUX (EURL)	PCB-BEQ	29	pg/g fat
HRGCMS (NRL SK)	PCDD/F-TEQ	0.22	pg/g fat
HRGCMS (NRL SK)	PCB-TEQ	0.24	pg/g fat
HRGCMS (EURL)	PCDD/F-TEQ	0.2	pg/g fat
HRGCMS (EURL)	PCB-TEQ	0.4	pg/g fat
HRGCMS (EURL)	Sum Ind. PCBs	27	ng/g fat

✓ What can be the origin of such a difference ?

- Other AhR DL-activity molecules ?
- Other (mixed)halogenated molecules ?

bromo compounds. We decided to go in that direction with the GCxGC-TOFMS system because we were not limited to measuring SIM or MRM signals anymore. Using the TOF, we now had the capability of also monitoring the brominated compounds. We might be limited by resolution, but it should be relatively straightforward to isolate the MS signal of the brominated compounds. We were able to highlight the presence of several brominated furans, including TBDF, one of the most toxic brominated compound. By performing semi-quantification of the recorded signal, and crossing the value with reported BEQ values, we estimated a possible BEQ contribution of this congener at a level close to 50 pg BEQ—most probably a direction to further investigate with proper method and standards to confirm that the high BEQ response was at least partly due to the presence of brominated furans. There is also the option of using ultra-high resolution with another instrument if the medium resolution shows limitations. Thus, our future plans include investigating both these approaches, which could be the best way to highlight the presence of these brominated compounds in these types of samples.

Summary

Our investigations have clearly shown that TOFMS analysis is a very exciting addition to the characterization of dioxins and related compounds using two-dimensional gas chromatography. There is still room for both high-resolution magnetic sector MS, and a triple-quadrupole MS, but they are definitely limited in their flexibility and their ability to monitor and identify unknown peaks that are not anticipated in your sample. However, the motivation for the testing labs will always be related to legislation. For that reason, the analytical community needs to see a return on investment, so even though there is obviously a need to get a better understanding of the problem from a public health perspective, there must be a business incentive to invest in the technique. So, the scope of the analytical work in our laboratory will be expanded to include the brominated compounds, which are typically recognized as being some of the "bad actors," but in addition, we also plan to investigate some of the mixed chloro and bromo compounds. This is the direction we will be taking our future studies using TOFMS technology and, in particular, to look at the toxicity impact of these compounds and how they bind with the Ah receptors.

Further Information

More information about this study, including more detailed references, can be found in the following publications:

- P-H. Stefanuto et al., Conference Paper, 38th International Symposium on Halogenated Persistent Organic Pollutants (Dioxin 2018), Kraków, Poland, Volume 80, August 2018.
- J-F. Focant et al., *J. Chromatog. A.* **1086** (1–2), 45–60 (2005).
- J-F. Focant, "GC×GC–TOFMS in Food and Feed Control: Going Beyond Dioxin Measurements," *LCGC* webcast, October 26, 2018 (available on demand), http://www.chromatographyonline.com/lcgc/measurements