

# Application News

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*Food Safety Analysis / GCMS-TQ8040*

## Analysis of Dioxins in Feed and Food Using GC-MS/MS as Confirmatory Method in Complying with EU Regulation

### □ Introduction

Polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are generally termed as dioxins<sup>1</sup>. It has been reported that dioxins accumulate in environment, plants and organisms due to their good chemical stability and good solubility in animal fats. Dioxins are probable carcinogens and possess a range of other toxic effects to humans<sup>2</sup>. The bioaccumulation greatly impacts humans who are on the top of food chain. Dioxins and other persistent organic pollutants (POPs) have been subjected to the Stockholm Convention, which obliges signatories to eliminate and minimize their sources. The analysis of dioxins in food and compound feed, the latter contributing to the dioxins content in food with animal origin, is essential. Gas chromatograph coupled with a high resolution mass spectrometry (GC-HRMS) was adopted first in determination of dioxins at trace levels for its high selectivity and sensitivity. Since June 2014, the EU regulation has included the use of tandem mass spectrometry system (GC-MS/MS) as a confirmatory method. This enhances the accessibility and scales down the costs of dioxins analyses<sup>3</sup>. In this study, Shimadzu's GCMS-TQ8040, a new model GC-MS/MS, was employed and evaluated for identification and quantification of the seventeen most toxic PCDD and PCDF congeners listed by the EU regulation. The analysis was carried out with mixed dioxin standards, as well as animal compound feed and animal-related food samples. The results show that Shimadzu's tandem quadrupole GCMS-TQ8040 fulfill all the requirements as stated in the EU regulation.

### □ Experimental

#### Instrumental and analytical conditions

A triple quadrupole GC-MS/MS system, GCMS-TQ8040 (Shimadzu Corporation, Japan) was employed in this work. The details of the system and analytical conditions are shown in Table 1.

#### Samples and chemicals

Calibration standards containing seventeen 2,3,7,8-chlorine substituted PCDD and PCDF congeners, as well as the corresponding <sup>13</sup>C-labelled internal standards and the surrogate standard, <sup>13</sup>C-1,2,3,4-TeCDD, were purchased from Cambridge Isotope Laboratories.

The types of samples that were tested include eggs, animal fats to animal compound feeds. The test samples which were weighed and spiked first with 2ng/mL labelled internal standard were extracted with the Power-Prep dioxin system (FMS Inc., USA). They were then subjected to clean-up using the DEXTech system (LCtech, Germany). Each cleaned-up sample was concentrated subsequently to 10µL and added with another 10µL of 200ng/mL surrogate standard (<sup>13</sup>C-1,2,3,4-TeCDD). The final concentrations of the labelled internal standards and surrogate standard are 100ng/mL in the 20µL of sample obtained.

Table 1: GC-MS/MS analytical conditions for dioxin analysis

<b>Instrumentation</b>	
GC-MS/MS system	GCMS-TQ8040
Auto Injector	AOC-20i+s
Column	Rxi-5Sil MS (60m x 0.25mm x 0.25µm)
Software	GCMSsolution Ver. 4.20
<b>Gas Chromatograph</b>	
Injection Condition	285°C, splitless mode
Injection Volume	2µL
Carrier Gas	Helium
Gas Flow Condition	Constant linear velocity mode Linear velocity 29.4cm/s Purge flow 5mL/min
Oven Temperature Programming	150°C (1min) →20°C/min to 220°C →2°C/min to 260°C (3min) →5°C/min to 320°C (8.5min)
<b>Mass Spectrometer</b>	
Ion Source Temperature	230°C
Interface Temperature	280°C
Solvent Cut Time	16min
Acquisition Mode	MRM

## □ Results and Discussion

### Requirements and criteria for GC-MS/MS

For unequivocal determination of dioxins in feed and food by using GC-MS/MS, several requirements have been amended in the commission regulation (EU) No. 589/2014. These modifications include the limit of quantification of individual congeners and new specific requirements for confirmatory methods. These, along with some other basic requirements, are evaluated in this study.

Three criteria have been established for GC-MS/MS analysis of dioxins<sup>3</sup>:

- (1) The resolution of the quadrupoles (Q1 & Q3) must be equal to or better than unit mass resolution.
- (2) The MRM method must monitor at least two specific precursor ions, each with specific transition product ion.
- (3) The maximum permitted tolerance of relative ion intensities is  $\pm 15\%$  for every MRM transition.

### Development of GC-MS/MS method

In method development on GCMS-TQ8040, the mass resolution of Q1 and Q3 was set to 0.9 to fulfill the first criterion. With this mass resolution, peaks which are unit mass apart can be separated explicitly, minimizing possible interferences to the analytes.

To meet the second criterion, two MRM transitions were used for each compound and its labelled internal standard. The optimized collision energies (CE) of the transitions of all compounds are compiled into Table 2.

In addition to retention time and MRM transitions, relative ion intensities (by area) of reference transition (T2) with respect to the target transition (T1) is used to enhance the reliability on confirming the analytes. The relative peak area ratio (T2 area over T1 area) is averaged from calibration standards. These values are summarized in Table 2. Only samples with relative ion intensities within  $\pm 15\%$  of the average value can be considered as an unmistakable identification of dioxins congeners.

Figure 1 shows the TIC of seventeen dioxins congeners, achieving excellent separation including the isomers. For example, for isomers 1,2,3,4,7,8-HxCDF and 1,2,3,6,7,8-HxCDF, it is required that peak-to-peak overlap must be less than 25%. It can be seen from Figure 1, their peaks are minimally overlapped (0.26% overlap). The resolution ( $R_s$ ) and peak overlap of two critical pairs are calculated and indicated in Figure 1.

### Performance of GC-MS/MS method

#### Calibration range & linearity

Calibration curves of five concentration levels for each congener were established, as illustrated in Table 3. The calibration ranges differ in accordance to the toxicity level of the dioxin congeners. The most toxic tetra-CDD and tetra-CDF congeners are calibrated from 0.5 to 200 ng/mL. Penta-, hexa- and hepta- congeners are calibrated from 2.5 to 1000 ng/mL. Octa-congeners are calibrated from 5 to 2000 ng/mL. All the seventeen congeners were calibrated with good linearity ( $R^2 > 0.998$ ). Three calibration curves of the two most toxic dioxin congeners and a congener with the lowest linearity are portrayed in Figure 2.

#### Limit of quantitation (LOQ)

The extremely low noise level of MRM chromatograms makes the calculation of signal-to-noise ratio (S/N) not feasible, which results in unreliable LOQ determination. Circumventing this difficulty, we follow the EU regulation which allows the use of calibration curve to determine LOQ instead. Accordingly, the LOQ is defined as **the lowest concentration level on a calibration curve that gives an acceptable and consistent deviation to the average relative response factor (RF) calculated for all levels on the calibration curve in each series of samples**. In other words, if the lowest concentration level has an RF within 30% of the mean RF of the calibration curve, the concentration at that level is the LOQ of the method. The results of calibration curves, mean RF and RSD of the lowest levels (LOQs) are summarized in Table 3.

Table 2: MRM transitions, collision energies and averaged relative intensity ratios of dioxins and <sup>13</sup>C-labelled internal standards.

Congener Type	PCDD					PCDF				
	Target transition 1, T1	CE (V)	Reference transition 2, T2	CE (V)	T2 area / T1 area (%)	Target transition 1, T1	CE (V)	Reference transition 2, T2	CE (V)	T2 area / T1 area (%)
tetra	319.90>256.90	24	321.90>258.90	24	95	303.90>240.95	33	305.90>242.95	33	95
<sup>13</sup> C-tetra	331.90>268.00	24	333.90>270.00	24	94	315.95>251.95	33	317.95>253.95	33	92
penta	355.90>292.90	25	353.90>290.90	25	77	339.90>276.90	35	337.90>274.90	35	79
<sup>13</sup> C-penta	365.90>301.90	25	367.90>303.90	25	80	351.90>287.90	35	349.90>285.90	35	80
hexa	389.80>326.90	25	391.80>328.80	25	67	373.80>310.90	35	375.80>312.90	35	65
<sup>13</sup> C-hexa	399.90>335.90	25	401.80>337.90	25	62	385.80>321.90	35	387.80>323.90	35	64
hepta	423.80>360.80	25	425.80>362.80	25	79	407.80>344.80	36	409.80>346.80	36	80
<sup>13</sup> C-hepta	435.80>371.80	25	437.80>373.80	25	79	419.80>355.90	36	421.80>357.90	36	79
octa	457.70>394.70	26	459.70>396.70	26	98	441.80>378.80	35	443.80>380.80	35	94
<sup>13</sup> C-octa	469.80>405.80	26	471.80>407.80	26	95	453.80>389.80	35	455.80>391.80	35	95

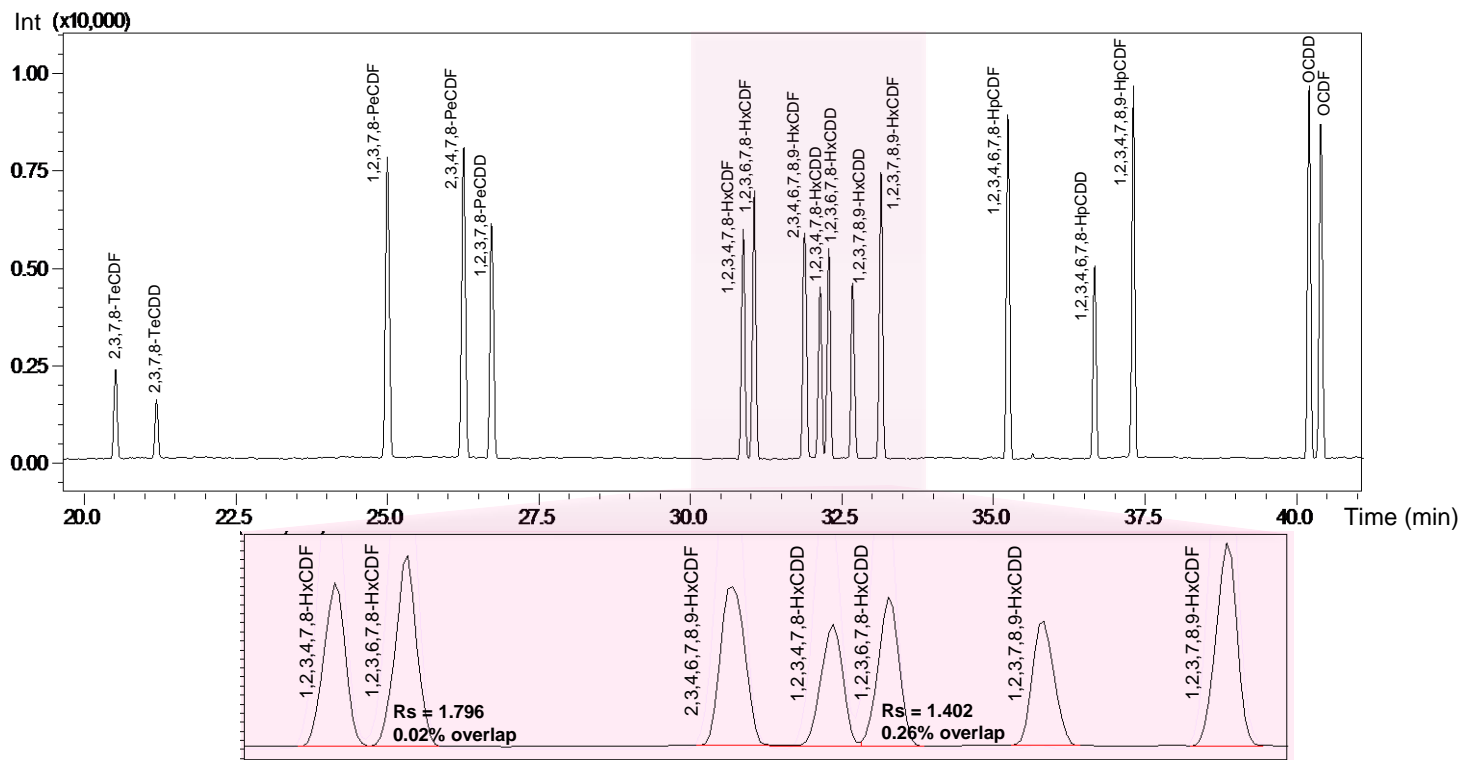


Figure 1: Total Ion Chromatogram of 17 dioxins congeners at 0.1ng/mL (TCDD/F), 0.5ng/mL (PeCDD/F, HxCDD/F & HpCDD/F), 1.0ng/mL (OCDD/F). Zoomed portion of the overlapping hexa-congeners group (30~33.8min) is displayed at bottom.

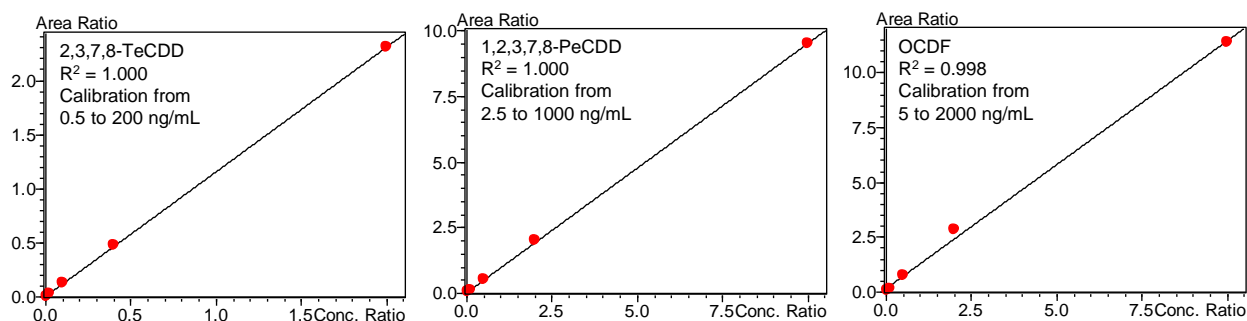


Figure 2: Calibration curves of the most toxic dioxin congeners and a congener with the least correlation coefficient

Table 3: Summary of calibration information using internal standard method and repeatability at lowest calibration level (n=10) for 17 regulated dioxins congeners

Compound name*	Calibration Range (ng/mL)	R <sup>2</sup>	Calibration Mean RF	Deviation of Lowest Conc. Level from Calibration Mean RF	%RSD of Lowest Level (n=10)
2,3,7,8-TeCDD	0.5 - 200	1.000	1.23	0.3%	8.1%
1,2,3,7,8-PeCDD	2.5 - 1000	1.000	1.03	4.2%	4.6%
1,2,3,4,7,8-HxCDD	2.5 - 1000	1.000	1.08	1.0%	5.8%
1,2,3,6,7,8-HxCDD	2.5 - 1000	1.000	1.08	1.7%	6.8%
1,2,3,7,8,9-HxCDD	2.5 - 1000	1.000	1.07	3.7%	3.4%
1,2,3,4,6,7,8-HpCDD	2.5 - 1000	1.000	1.11	1.3%	6.0%
OCDD	5.0 - 2000	0.999	1.20	10.7%	5.2%
2,3,7,8-TeCDF	0.5 - 200	1.000	1.11	3.0%	6.1%
1,2,3,7,8-PeCDF	2.5 - 1000	1.000	1.14	7.5%	4.5%
2,3,4,7,8-PeCDF	2.5 - 1000	1.000	1.06	5.5%	3.0%
1,2,3,4,7,8-HxCDF	2.5 - 1000	1.000	1.13	5.6%	5.5%
1,2,3,6,7,8-HxCDF	2.5 - 1000	1.000	1.02	9.5%	3.0%
2,3,4,6,7,8-HxCDF	2.5 - 1000	1.000	1.02	0.4%	5.4%
1,2,3,7,8,9-HxCDF	2.5 - 1000	1.000	1.02	6.2%	6.2%
1,2,3,4,6,7,8-HpCDF	2.5 - 1000	1.000	1.16	5.3%	7.5%
1,2,3,4,7,8,9-HpCDF	2.5 - 1000	1.000	1.08	1.9%	6.7%
OCDF	5.0 - 2000	0.998	1.41	11.3%	4.0%

\*Abbreviations: "Te" = tetra; "Pe" = penta; "Hx" = hexa; "Hp" = hepta; "O" = octa; "CDD" = chlorodibenzodioxin; "CDF" = chlorodibenzofuran

#### Detection sensitivity (LOD)

The sensitivity of the GC-MS/MS method was examined with low concentration of mixed standards. As depicted in Figure 1, a concentration level of 5 times lower than the lowest level that was used for making calibration curves was analyzed. All the seventeen dioxin congener peaks were detected clearly. This proves that the GC-MS/MS employed and the MRM method used are capable of detecting low concentrations of dioxins as per the current regulation requirement.

The LODs of the method for the 17 dioxins are estimated to be at 1/3~1/5 levels of their LOQs. The LOQs are concentrations of the lowest calibration standards, i.e. 0.5pg/μL to 5pg/μL, and the LODs are thus expected to be around 0.1pg/μL to 1.6pg/μL. This is in alignment to the EU regulation which states that the detectable quantities of dioxins have to be in the upper femtogram ( $10^{-15}$ g) range<sup>3</sup>.

#### Repeatability

To test for method precision for low concentration samples, ten consecutive runs of the lowest calibration level mixed standards were performed. The %RSD of the peak area ratios with respect to the internal standards were less than 10% for all compounds (see Table 3). This satisfies the analytical criterion in which within-laboratory reproducibility of a confirmatory method has to be less than 15%<sup>3</sup>.

#### Sample Analysis & TEQ calculation

It is well known that each of the regulated dioxin congeners exhibits different toxicity to humans, and TeCDD is the most lethal of all congeners. Toxic equivalent factors (TEF) of the regulated dioxins have been established and used to express their relative toxicities in comparison to TeCDD. Furthermore, toxic equivalent quantity (TEQ)<sup>1</sup>, a quantifiable amount expressed as the TeCDD toxicity for a mixed-dioxins containing sample, can be calculated from the concentrations of dioxins and their TEF values. The TEF values of the regulated dioxins are listed in Table 4.

The developed MRM method was applied to actual food and feed samples for detection and quantitation of dioxins. First, internal standard peaks were identified within the required criteria. The recoveries of individual internal standards in reference to the surrogate standard added ranged from 60% to 120%. It is worth noting that for those congeners which contribution to the TEQ value is less than 10% (mainly the hepta- and octa- congeners), lower or higher recoveries beyond the above range are acceptable<sup>3</sup>. If the samples have recovery within the stated range, they are regarded as having negligible matrix effect and are then quantified.

Table 4: Toxic Equivalent Factors of dioxin congeners and TEQ calculation of an actual sample from GC-MS/MS data

Congener	TEF value	Conc. in sample (ng/mL)	TEQ weight in 20μL sample (ng)	TEQ in 5g sample (ng/kg)
2,3,7,8-TeCDD	1	0.913	0.01826	3.652
1,2,3,7,8-PeCDD	1	5.622	0.11244	22.488
1,2,3,4,7,8-HxCDD	0.1	6.081	0.01216	2.432
1,2,3,6,7,8-HxCDD	0.1	4.463	0.00893	1.785
1,2,3,7,8,9-HxCDD	0.1	3.242	0.00648	1.297
1,2,3,4,6,7,8-HpCDD	0.01	4.509	0.00090	0.180
OCDD	0.0003	7.049	0.00004	0.008
2,3,7,8-TeCDF	0.1	0.966	0.00193	0.386
1,2,3,7,8-PeCDF	0.03	5.176	0.00311	0.621
2,3,4,7,8-PeCDF	0.3	5.769	0.03461	6.923
1,2,3,4,7,8-HxCDF	0.1	5.793	0.01159	2.317
1,2,3,6,7,8-HxCDF	0.1	5.266	0.01053	2.106
2,3,4,6,7,8-HxCDF	0.1	5.428	0.01086	2.171
1,2,3,7,8,9-HxCDF	0.1	5.190	0.01038	2.076
1,2,3,4,6,7,8-HpCDF	0.01	4.718	0.00094	0.189
1,2,3,4,7,8,9-HpCDF	0.01	4.214	0.00084	0.169
OCDF	0.0003	10.040	0.00006	0.012
<b>Sum</b>				<b>48.814</b>

Table 4 also illustrates the TEQ results of a found positive sample and the details of the sum TEQ calculation. In short, the measured concentration of a dioxin congener in the sample solution is multiplied by its TEF value and the volume (20μL) to obtain the TEQ in weight (ng). This TEQ in weight (ng) is then divided by the actual sample amount (weight) before sample pretreatment (5g). The sum TEQ of the sample (48.814ng/kg) is obtained by summing up the individual TEQ value.

The maximum permitted content of dioxins expressed in TEQ differs from sample types. For instance, the limit in food with animal origin is 1.50 ng per kg of animal fat (including milk fat and egg fat) and 0.75 ng per kg of compound feed as well as other land animal products (including eggs and egg products)<sup>4</sup>. The total TEQ of the found positive sample illustrated in Table 4 is significantly higher than the maximum permitted content.

#### Conclusions

The Shimadzu GCMS-TQ8040 and the MRM method established are capable of detection and quantitation of seventeen regulated dioxins with high sensitivity and selectivity. The results show that the method and system indeed comply with the requirements of the EU regulation for confirmatory analysis of dioxins in feed and food.

#### References

1. EU regulation 152/2009.
2. Toxicological Sciences 93(2), 223-241 (2006).
3. EU regulation 589/2014.
4. EU regulation 277/2012.