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SCIENTIFIC SERVICES

### DETERMINATION OF CHLORINATED DIOXINS/FURANS IN MAINSTREAM CIGARETTE SMOKE USING GAS CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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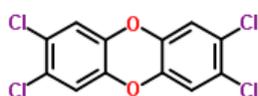
#### Summary

Chlorinated dioxins and furans have been cited by the United States Food and Drug Administration (FDA) as harmful and potentially harmful constituents (HPHC) in tobacco smoke. It is the only constituent on the FDA list which is not a specific compound; rather it refers to a sub-group of compounds consisting of tetra- through octa-chlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF). By convention they are usually referred to by the generic term 'dioxins'. They are anthropogenic chemicals formed from combustion/pyrolysis and manufacturing processes. The World Health Organisation (WHO) has defined 17 dioxin congeners as significantly toxic, and adopted a system of reporting the total toxic equivalence (TEQ) based on the mass of each component multiplied by a toxic equivalence factor (TEF). The United States Environmental Protection Agency (US EPA) has set a threshold for safe dioxin exposure at 0.7 pg TEQ/kg body weight per day.

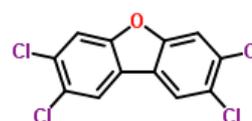
A clean-up procedure using the 'Dioxin Prep System' followed by analysis using Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS) was applied. Dioxins were not detected (<4.3 pg TEQ/cig) in Kentucky (K3R4F) and CORESTA (CM7) reference cigarettes under ISO 3308 and Health Canada Intense (HCI) smoking conditions. Transfer rates were evaluated by spiking 500 pg of each component into the tobacco rod and collecting the smoke condensate onto a Cambridge filter (CF) pad. Reproducible measurements were obtained with recoveries of 4% to 11% (ISO) and 9 to 27% (HCI). Recoveries of the <sup>13</sup>C isotope labelled internal standards were between 74.5% and 125.7%.

## Dioxins Structure and Toxic Activity

Chlorinated dioxins and furans (dioxins) consist of a polychlorinated dibenzo structure bridged by either two oxygen atoms (PCDD) or one oxygen atom (PCDF). Toxic activity is mediated through the aryl hydrocarbon receptor (AHR) which has a high affinity for dioxins containing lateral chlorines at the 2-, 3-, 7-, and 8- positions (Figure 1). WHO and other organisations cite 2,3,7,8-tetrachlorodibenzodioxin (2378-TCDD) as having the most toxic activity and the reference by which toxic equivalence (TEQ) is measured (Table 1). TEQ provides a toxicity-weighted concentration of the 17 congeners to reflect the different levels of associated risk to human health. These risks include skin disorders, cardiovascular disease, developmental and reproductive toxicity, immunotoxicity, and cancer [1]. The effect of dioxin exposure caused by smoking has not been studied, to our knowledge. However, the highly lipophilic nature of these chemicals indicates that any amount of exposure could lead to incorporation into the biomass and prolonged exposure to bioaccumulation. The US EPA set its threshold for safe dioxin exposure at 0.7 pg TEQ/kg body weight per day [2] and is based on more recent data, while the WHO limit exposure level is slightly higher at 1-4 pg TEQ/kg body weight per day. .



2,3,7,8-Tetrachlorodibenzo-p-dioxin (TEF = 1)



2,3,7,8-Tetrachlorodibenzo[b,d]furan (TEF = 0.1)

Figure 1. Structures of poly-chlorinated dioxins (PCDD) and furans (PCDF)

Table 1. Toxic Equivalence Factor (TEF) Values defined by the World Health Organisation (2005)

PCDD	TEF
2378-TCDD	1
12378-PCDD	1
123478-HxCDD	0.1
123678-HxCDD	0.1
123789-HxCDD	0.1
1234678-HpCDD	0.01
OCDD	0.0003
PCDF	
2378-TCDF	0.1
12378-PCDF	0.03
23478-PCDF	0.3
123478-HxCDF	0.1
123678-HxCDF	0.1
234678-HxCDF	0.1
123789-HxCDF	0.01
1234678-HpCDF	0.01
1234789-HpCDF	0.01
OCDF	0.0003

## **Standard Analytical Methods**

Standard methods are available for the analysis of dioxins arising from industrial contamination of various matrices such as soil, waste water, fly ash, and biological tissue [3-7]. They are 'performance-based' methods which provide a compilation of protocols and a set of criterion but require adaptation to the type of matrix under investigation. The essential aspects of these methods are isotope dilution of the matrix using <sup>13</sup>C labelled dioxin analogues followed by solid-phase extraction (SPE) usually involving multiple stages. Analysis is usually performed using gas chromatography (GC) coupled with high-resolution (>10,000) mass spectrometry (HRMS). Method detection limits (MDL) in the parts per trillion (PPT) range can be achieved depending on the type of matrix. Recent advances in low-resolution tandem mass spectrometers (MS/MS), such as triple quadrupole, have enabled very low detection limits to be realised and potentially offer an alternative to HRMS.

## **Dioxins in Cigarette Smoke**

Dioxins have reportedly been detected in the cigarette mainstream smoke (MSS) of certain commercial brands [8-9]. However, the data available is limited. The most detailed study was carried out by Ball and co-workers who published data from ten German brands [8]. The analyses were performed using HRMS. Certain dioxin congeners were detected in all of the brands tested; however, the most toxic, 2378-TCDD and 12378-PCDD, were not detected. Only two congeners, 123478-HpCDD and OCDD, were clearly resolved from the matrix with concentrations between 0.8 and 5.4 pg/cig. Dioxins are ubiquitous in the environment and it is reasonable to assume their presence in tobacco smoke; however, standards relating to industrial emissions have improved considerably since these studies were made. The source of these chemicals could be environmental contamination or combustion/pyrolysis of dioxin precursors found in the tobacco and/or paper.

## **Aims & Objectives**

The evaluation of a clean-up procedure developed by Maeoka et al. [10] and analysis using GC-MS/MS is presented to determine whether dioxins are present at detectable levels in Kentucky and CORESTA reference cigarettes. An assessment of the reproducibility of the method and other validation parameters are also presented.

## **Principal of Operation**

### ***Dioxin Prep System***

The clean-up procedure was performed using a two-stage procedure (Figure 2). A multi-layer column consisting of four activated silica gel layers removes much of the polar interference such as lipids and bases while allowing dioxins to pass through. A dual-layer carbon reversible column consisting of low- and high-surface area charcoal layers retains dioxins from a non-polar solvent, which can then be eluted with a polar solvent such as toluene. The columns are connected using PTFE unions during the load cycle.

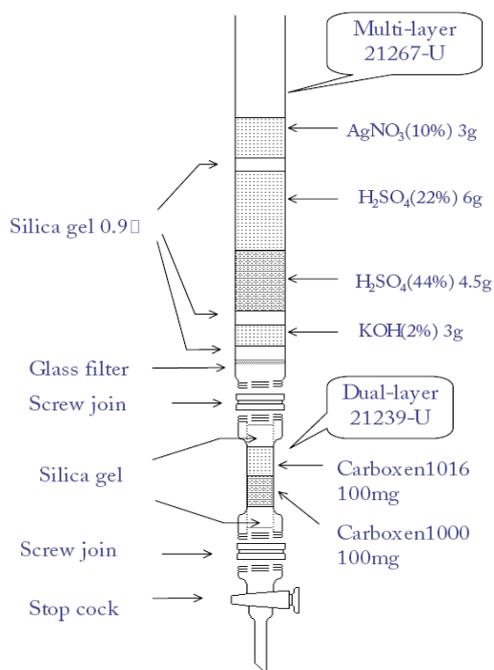


Figure 2. Multi-layer silica gel column and dual-layer carbon column

### **GC-MS/MS**

Tandem mass spectrometry is a unit-resolution three stage mass analyser. The principal of the operation is two mass analysers operate in tandem which eliminates matrix interference and subsequently lowers the baseline. Pre-cursor ions are resolved in the first quadrupole (Q1). A collision cell (Q2) placed after Q1 causes further ion fragmentation to occur. This is known as multiple reaction monitoring (MRM). The product ions are then resolved in the second quadrupole (Q3), which produce a signal response amplified using an electron multiplier (EM).

## Experimental

### Materials and Equipment

Cigarettes were smoked using a 20-port Rotary smoking machine (RM20/CS or RM20D, Borgwaldt KC, Hamburg, Germany). Kentucky reference cigarettes (K3R4F) were provided by the Kentucky Tobacco Research and Development Center (Lexington, KY, USA) and CORESTA monitor cigarettes (CM7) by Cerulean (Milton Keynes, UK). SPE columns and equipment were supplied by Supelco (Sigma, St. Louis, MO, USA). Certified reference standards were provided by Accustandard (New Haven, CT, USA) and Cambridge Isotope Laboratories (Tewksbury, MA, USA). GC-MS/MS equipment was an Agilent 7890GC / 7000B MS (Agilent Technologies, Santa Clara, CA, USA). Separation was performed using a Restek Rxi-5ms column (30m x 0.25mm x 0.25µm) (Restek, Bellefonte, PA, USA). Toluene (PCB grade), hexane and dichloromethane (HPLC grade), and nonane (analytical standard grade) were supplied by Sigma. TurboVap® II Concentrator was supplied by Biotage (Cardiff, UK).

### Calibration Standards

Calibration standards were made up in 0.5 ml toluene in the range of 0.10 – 50 ng/ml (tetra-, penta-, hexa-) and 0.25 – 50 ng/ml (hepta-, octa-) with an internal standard (ISTD) concentration of 50 ng/ml. One labelled analogue ( $^{13}\text{C}_{12}$ ) from each homologue group was added (Table 2). Therefore, ten congeners were quantified using exact analogues and seven using surrogates.

Table 2. Dioxin analytes with corresponding exact or surrogate analogues used as internal standards

ISTD Correlation	
PCDD	$^{13}\text{C}$ Analogue
2378-TCDD	2378-TCDD $^{13}\text{C}_{12}$
12378-PCDD	12378-PCDD $^{13}\text{C}_{12}$
123478-HxCDD	123478-HxCDD $^{13}\text{C}_{12}$
123678-HxCDD	
123789-HxCDD	
1234678-HpCDD	1234678-HpCDD $^{13}\text{C}_{12}$
OCDD	OCDD $^{13}\text{C}_{12}$
PCDF	
2378-TCDF	2378-TCDF $^{13}\text{C}_{12}$
12378-PCDF	12378-PCDF $^{13}\text{C}_{12}$
23478-PCDF	
123478-HxCDF	123478-HxCDF $^{13}\text{C}_{12}$
123678-HxCDF	
234678-HxCDF	
123789-HxCDF	
1234678-HpCDF	1234678-HpCDF $^{13}\text{C}_{12}$
1234789-HpCDF	
OCDF	OCDF $^{13}\text{C}_{12}$

## Smoking and Extraction Procedure

Reference cigarettes were smoked under ISO 3308 and Health Canada Intense (HCI) smoking conditions. The smoke condensate from 20 cigs (ISO) and ten cigs (HCI) was trapped onto 92 mm Cambridge filter (CF) pads. The CF pads were spiked with 50 µl ISTD solution (0.5 µg/ml; nonane). The pads were extracted into 2 x 50 ml hexane using a wrist-action shaker.

## Clean-up

Multi-layer dioxin columns were conditioned with 200 ml hexane. Dual-layer reversible carbon columns were conditioned with 20 ml toluene then 40 ml hexane. The columns were connected using PTFE unions and the sample extracts loaded onto the column. After the sample extract had passed through the multi-layer column it was detached and replaced with an empty column to be used as a solvent reservoir. The dual-layer column was rinsed with 10 ml hexane (3% dichloromethane). Dioxins were eluted with 50 ml toluene and collected. The eluent was concentrated to 0.5 ml using a TurboVap (60°C; ~14psi). The sample was transferred to a crimped top amber autosampler vial for analysis.

## Spiked Tobacco Rods

The transfer rates were evaluated by spiking K3R4F tobacco rods with known quantities of dioxins. 1 µl mixed working stock standard (0.5 µg/ml; toluene) was injected into the front of the tobacco rod to a depth of 20 – 30 mm. Therefore, each cigarette contained 500 pg of each dioxin congener. The spiked rods were smoked as previously described under ISO 3308 and HCI conditions.

## Spiked Pad Recoveries

K3R4F reference cigarettes were smoked under ISO and HCI conditions. 5 µl or 40 µl from a mixed standard solution (25 ng/ml; toluene) was spiked onto the pad prior to clean-up. Multiple analyses were performed to evaluate limit of detection/quantification (LOD/LOQ) and method precision, respectively.

## GC-MS/MS

Inlet:	280°C; 1µl splitless (1 min)
Carrier:	Helium; 1.2 ml/min constant flow
Oven:	90°C (1 min), 40°C/min to 200°C (0.5 min), 3°C/min to 310°C (3 min)
Total RT:	43.9 min
Transfer Line:	300°C
Source:	280 °C (EI; 70 eV)

Quadrupoles 150°C  
 Collision Cell Gas 1: Helium (2.3 ml/min)  
 Collision Cell Gas 2: Nitrogen (1.5 ml/min)  
 Collision Energy: Optimised to compound (25 – 40V)

## Results and Discussion

Dioxins were not detected in K3R4F or CM7 reference cigarettes under ISO or HCl smoking conditions. LOD/LOQ limits were determined by empirical precision based on ten determinations, smoked under ISO conditions, of a K3R4F spiked pad recovery (6.3 pg/cig equivalent for each congener) (Table 3). Individual levels were calculated for each congener and an overall LOD determined (4.3 pg TEQ /cig). Cigarettes were also smoked with the filters removed and also determined to be <LOD. Although the LOD was based on an empirical measurement, observation has confirmed they are not present at the lower end of the dynamic range of the method. Clear resolution was observed for each component with no matrix interference (Figure 3).

Table 3. LOD/LOQ values calculated from pad spike recoveries (6.3 pg/cig equivalent)

PCDD	mean	SD	LOD (SD*3)	LOQ (SD*10)	LOD TEQ pg/cig	LOQ TEQ pg/cig
2378-TCDD	7.3	0.33	1.0	3.3	1.00	3.34
12378-PCDD	7.0	0.55	1.6	5.5	1.65	5.49
123478-HxCDD	7.6	1.28	3.8	12.8	0.38	1.28
123678-HxCDD	7.0	0.83	2.5	8.3	0.25	0.83
123789-HxCDD	7.0	0.55	1.7	5.5	0.17	0.55
1234678-HpCDD	6.5	1.08	3.2	10.8	0.03	0.11
OCDD	7.3	0.69	2.1	6.9	0.0006	0.0021
<b>PCDF</b>						
2378-TCDF	7.3	0.27	0.8	2.7	0.08	0.27
12378-PCDF	7.4	0.48	1.4	4.8	0.04	0.14
23478-PCDF	7.0	0.27	0.8	2.7	0.24	0.80
123478-HxCDF	7.5	0.68	2.0	6.8	0.20	0.68
123678-HxCDF	6.7	0.29	0.9	2.9	0.09	0.29
234678-HxCDF	6.4	0.34	1.0	3.4	0.10	0.34
123789-HxCDF	6.2	0.24	0.7	2.4	0.01	0.02
1234678-HpCDF	7.4	0.56	1.7	5.6	0.02	0.06
1234789-HpCDF	7.0	1.16	3.5	11.6	0.03	0.12
OCDF	7.0	1.05	3.2	10.5	0.0009	0.0032
Overall TEQ pg/cig					4.3	14.3

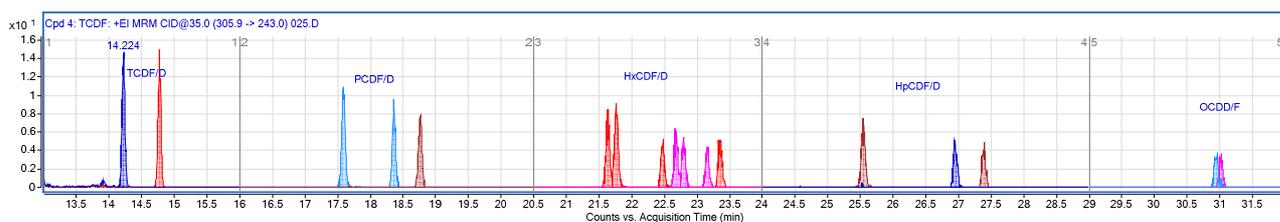


Figure 3. Overlaid MRM chromatogram of a K3R4F spiked extract (6.3 pg/cig equivalent)

Transfer rates were evaluated by ten determinations under both ISO and HCl conditions (Table 4). The total amount of dioxins spiked onto the tobacco rods in terms of toxic equivalence was 1535.3 pg TEQ /cig. The TEQ recoveries under HCl (23.9%) were considerably higher than ISO (9.5%) conditions which reflect the higher TPM values (~40 mg K3R4F (HCl); ~10 mg K3R4F (ISO)). Recoveries of congeners quantified using exact ISTD analogues (mean 9.4% ISO; mean 23.8% HCl) were significantly higher than those quantified with surrogates (mean 6.6% ISO; mean 16.6% HCl) which was most likely due to some isomeric fractionation. Crucially the most toxic species, 2378-TCDD and 12378-PCDD were in the former category. The remainder of the dioxins are most likely to have either been lost to the sidestream smoke or captured in the filter tip. However, some thermal decomposition may have also occurred. An estimate of uncertainty (U) for each congener was derived from the relative standard deviation (RSD) with a coverage factor (k = 2). It is noted that dioxins contained within a small area of the tobacco do not truly reproduce actual contaminated tobacco. A minimal amount (1 ml) of solvent was used for spiking the rods to avoid alteration of the tobacco matrix. Further analyses of spiked rods were performed at 191.9, 383.8 and 767.7 pg TEQ /cig (Table 5). Even at the lowest spiked level most dioxin congeners were present at detectable levels. At this level dioxins were quantified at 16.8 pg TEQ/cig.

Table 4. Transfer rates from tobacco rods spiked with 500 pg of each congener

Regime	ISO 3308 (n = 10)			HCl (n = 10)		
	Mean (pg/cig)	Recovery	U (k = 2)	Mean (pg/cig)	Recovery	U (k = 2)
<b>PCDD</b>						
2378-TCDD	55.5	11.1%	22.3%	135.7	27.1%	14.0%
12378-PCDD	51.3	10.3%	18.3%	131.5	26.3%	17.1%
123478-HxCDD	45.7	9.1%	16.5%	116.0	23.2%	19.3%
123678-HxCDD	44.8	9.0%	17.2%	110.1	22.0%	16.0%
123789-HxCDD	33.7	6.7%	32.1%	82.3	16.5%	20.3%
1234678-HpCDD	43.3	8.7%	21.6%	109.8	22.0%	13.7%
OCDD	45.8	9.2%	32.5%	110.2	22.0%	24.6%
<b>PCDF</b>						
2378-TCDF	53.0	10.6%	15.0%	136.0	27.2%	9.4%
12378-PCDF	51.6	10.3%	16.0%	128.2	25.6%	11.0%
23478-PCDF	25.5	5.1%	25.9%	61.4	12.3%	48.6%
123478-HxCDF	45.1	9.0%	17.3%	117.6	23.5%	8.4%
123678-HxCDF	47.6	9.5%	28.4%	129.6	25.9%	34.1%
234678-HxCDF	19.3	3.9%	21.2%	46.0	9.2%	38.0%
123789-HxCDF	23.8	4.8%	17.9%	61.5	12.3%	21.8%
1234678-HpCDF	44.2	8.8%	16.2%	111.5	22.3%	18.9%
1234789-HpCDF	35.9	7.2%	22.1%	90.7	18.1%	36.7%
OCDF	35.8	7.2%	23.5%	91.8	18.4%	20.6%
Total Dioxins pg TEQ/cig	146.5	9.5%		367.0	23.9%	

Table 5. Transfer rates from tobacco rods spiked with 63, 125 and 250 pg of each congener

Regime	ISO 3308			HCl
	63 pg/cig	125 pg/cig	250 pg/cig	125 pg/cig
<b>PCDD</b>				
2378-TCDD	8.4	9.1	33.7	30.2
12378-PCDD	<5.5	8.6	27.6	23.8
123478-HxCDD	6.8	14.8	28.8	23.1
123678-HxCDD	<LOD	14.6	25.2	20.2
123789-HxCDD	<5.5	15.4	8.7	22.5
1234678-HpCDD	<LOD	5.7	24.9	24.6
OCDD	12.5	13.3	30.2	35.2
<b>PCDF</b>				
2378-TCDF	8.7	10.5	30.8	25.0
12378-PCDF	6.8	10.9	28.5	24.8
23478-PCDF	<2.7	8.5	7.6	16.8
123478-HxCDF	<6.8	17.6	23.8	24.9
123678-HxCDF	5.0	16.0	24.8	22.8
234678-HxCDF	<LOD	<3.4	4.6	13.1
123789-HxCDF	<2.4	3.1	11.0	14.0
1234678-HpCDF	<5.6	10.0	24.6	22.0
1234789-HpCDF	<11.6	9.5	16.4	28.7
OCDF	<LOD	7.9	19.0	19.8
Total Dioxins pg TEQ/cig	16.8	30.2	79.8	75.9
Recovery (%)	8.8	7.9	10.4	19.8

Inter-and intra-day precision was evaluated under ISO and HCl smoking conditions. The relative recoveries were determined equivalent to 50 pg/cig (ISO) and 100 pg/cig (HCl) (Table 6). The standard error (SE) and the intraday precision (CV.r) were derived from multiple determinations performed in triplicate. This data was plotted onto control charts and used as a monitor control (Figure 4). Relative extraction efficiencies were between 89.1 to 112.7% (ISO) and 85.3 to 104.6% (HCl). Noticeable bias was observed for 234678-HxCDF. Although the overall recoveries were 89.1% (ISO) and 85.3% (HCl), three determinations were <80% of their nominal value under both smoking regimes.

Table 6. Inter- and Intra- Day Precision

Regime	ISO 3308 (n = 11)			HCl (n = 9)		
	Mean (pg/cig)	SE (%)	CV.r (%)	Mean (pg/cig)	SE (%)	CV.r (%)
<b>PCDD</b>						
2378-TCDD	50.7	5.2%	2.5%	100.6	4.1%	3.4%
12378-PCDD	49.0	7.8%	4.3%	98.3	3.9%	4.9%
123478-HxCDD	51.8	6.0%	4.3%	100.7	8.7%	4.0%
123678-HxCDD	49.1	6.8%	3.7%	95.9	4.1%	6.5%
123789-HxCDD	49.6	5.8%	6.6%	98.2	9.7%	7.0%
1234678-HpCDD	49.0	3.9%	5.8%	98.8	5.4%	4.6%
OCDD	52.7	6.0%	5.2%	104.5	8.0%	9.4%
<b>PCDF</b>						
2378-TCDF	49.3	5.2%	2.8%	98.5	3.9%	4.0%
12378-PCDF	49.3	6.0%	3.0%	97.3	3.1%	4.5%
23478-PCDF	46.5	7.2%	4.9%	89.4	6.3%	4.9%
123478-HxCDF	49.8	4.9%	2.8%	98.0	6.5%	4.6%
123678-HxCDF	49.7	8.5%	6.0%	96.3	7.0%	7.5%
234678-HxCDF	44.5	12.6%	7.0%	85.3	8.0%	9.0%
123789-HxCDF	47.0	8.6%	5.5%	90.5	10.3%	1.9%
1234678-HpCDF	48.5	5.4%	3.8%	98.6	5.3%	6.1%
1234789-HpCDF	56.4	3.5%	3.2%	104.6	6.6%	6.2%
OCDF	47.9	6.8%	8.6%	96.9	5.8%	8.1%

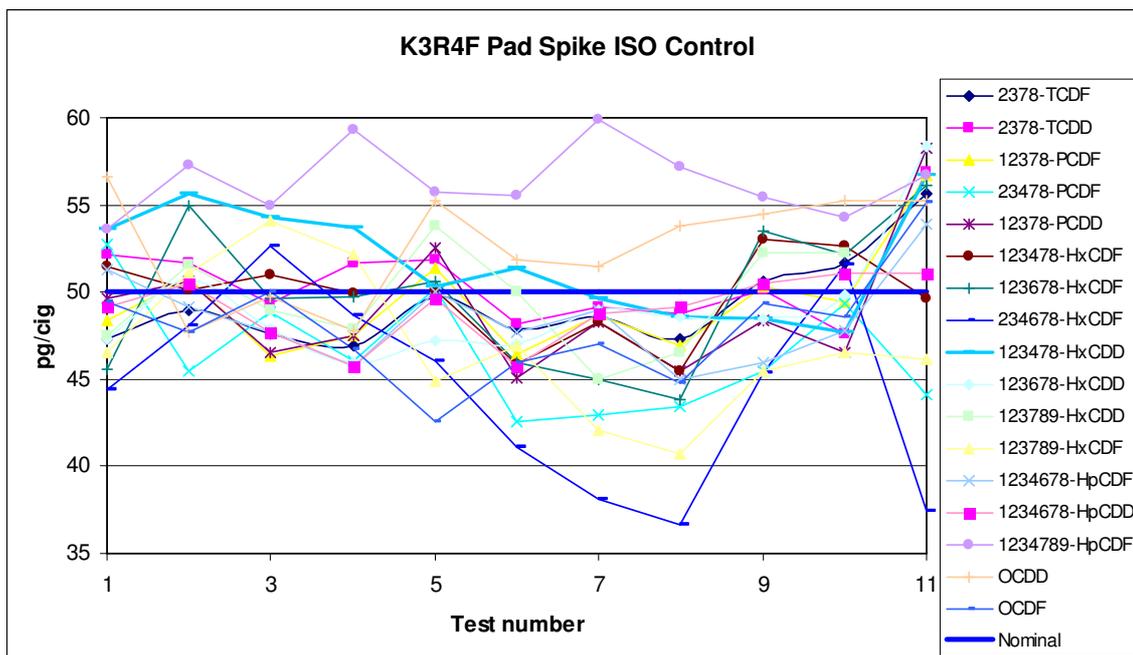


Figure 4. Inter-Day Precision Control Chart (ISO 3308)

Recoveries of the  $^{13}\text{C}$  isotope labelled ISTDs were evaluated by comparison of the signal response from 3 x calibration levels against 3 x samples. Recoveries of between 74.5% and 125.7% were obtained which is consistent with published data [4].

## Conclusion

Dioxins were not detected in K3R4F or CM7 reference cigarettes. The reported limit of detection (4.3 pg TEQ /cig) is an empirical value but has been supported by observation of the data. Dioxins have been measured, by this method, as low as 16.8 pg TEQ /cig and there is certainly scope to measure >1 order of magnitude lower. The results suggest that dioxins are not ordinarily produced in the combustion/pyrolysis of certain types of tobacco at levels that would exceed the US EPA safe limit (0.7 pg TEQ/kg body weight per day), assuming the smoker was not exposed to other sources of dioxins<sup>1</sup>. However, contaminated tobacco will produce measurable quantities in the smoke condensate and it is reasonable to infer that tobacco contaminated with dioxin pre-cursors (e.g. chlorophenol) may produce dioxins.

The clean-up using the Dioxin Prep System was shown to effectively remove the matrix interference and clear chromatographic resolution was observed for all of the congeners.

The removal of the matrix background is the key to low detection levels and because triple quadrupole MS is a dynamic system, it is possible to apply more voltage to the EM and further increase the sensitivity. Although there is a 'trade-off' as increasing the gain voltage does shorten the lifetime of the EM, replacement is required periodically anyway and the cost is roughly comparable to one vial of <sup>13</sup>C isotope labelled dioxin. Triple quadrupole MS offers a viable alternative to HRMS with comparable detection limits.

1. The study was carried out on American blend and Virginia cigarettes. Essentra has the testing machinery and expertise at its Scientific Services laboratory to measure dioxins and furans at very low (less than 100 picograms/cigarette) levels.

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