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PTV-LV-GC/MS/MS as screening and complementary method to HRMS for the monitoring of dioxin levels in food and feed

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Abstract

Recent developments in trapping efficiency inside ion trap mass spectrometer permitted to lower instrument detection limit (IDL). An IDL of 200 fg μl^{-1} injected with a signal-to-noise ratio of 5:1 for tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained by gas chromatography coupled to a quadrupole ion storage mass spectrometer in tandem mode (GC/MS/MS). Coupling large volume programmable temperature vaporizer (PTV-LV) injection to GC/MS/MS provides an alternative and complementary method to classical splitless-GC injection connected to high-resolution mass spectrometry (splitless-GC/HRMS) method for dioxin monitoring in food and feed.

An injection volume of 10 μl was found to be the best compromise between the sensitivity requirements and the robustness required for a high throughput method. PTV-LV-GC/MS/MS and Splitless-GC/HRMS were compared by performing analysis on five different matrices such as beef fat, yolk eggs, milk powder, animal feed and serum samples covering a concentration range of two orders of magnitude (i.e. 0.2–25 ng WHO-TEQ kg^{-1}). An analysis of variance (ANOVA) was carried out. Fisher tests pointed out that the method effect for all the 2,3,7,8 congeners was not significant, indicating that the null hypothesis (H_0: $\mu_1 = \mu_2 = \ldots = \mu_n$) was not rejected. Moreover, the interaction effects between methods and matrices were not significant for most of the 2,3,7,8 congeners. However, three congeners (2,3,7,8-TCDF; 1,2,3,4,7,8-HxCDD and 1,2,3,4,6,7,8-HpCDD) were characterized by *P*-values lower than the significance level ($\alpha = 0.05$). In toxic equivalence (TEQ), the study showed that no significant bias was observed between the two methods. Consequently, PTV-LV-GC/MS/MS is an attractive technique and can be used as a cost effective complementary method to HRMS for dioxin levels monitoring in food and feed.

Keywords: Programmable temperature vaporization injector; Large volume injection; Tandem mass spectrometry; Polychlorinated dibenzo-*p*-dioxin; Dibenzofuran; Food; Feed

1. Introduction

Mass spectrometry techniques are useful tools to provide the selectivity and the sensitivity required to analyse polychlorodibenzo-*p*-dioxin/furan (PCDD/F) congeners in foodstuffs. High-resolution gas chromatography coupled to high-resolution mass spectrometry (HRGC–HRMS) is considered since a long time as the reference method. This technique, also called the confirmatory method, can provide reliable dioxin results at sub-parts-per-trillion (ppt) levels. After several dioxin crises in the food chain in Europe, the European Union (EU) implemented comprehensive regulation on foodstuffs and feedingstuffs. Large monitoring programs to test food and feed have been launched and it is foreseen in the legislation to involve screening methods to select samples containing dioxin levels close to regulatory limits. Only those samples with a significant level of dioxin are confirmed by HRGC–HRMS [1]. The approach is sounded because more than 95 % of the samples randomly controlled are compliant with regulatory limits.

Among the screening methods, tandem mass spectrometry (MS/MS or more generally MS^n) using a low resolution quadrupole ion storage mass spectrometer is a very selective technique, its use in the dioxin food analysis field started in the nineties [2,3]. The technique provides good selectivity in MS^2 mode but the sensitivity cannot be compared with the specifications provided by the suppliers of high-resolution sector instruments (i.e. 100 fg injected with a signal-to-noise ratio of 100:1 for 2,3,7,8-TCDD in specific conditions). In 1994, Plombey et al. [4] already reported

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a value of 500 fg injected (with signal-to-noise ratio of 5:1) for the detection of 2,3,7,8-TCDD by ion trap MS/MS technique. More recently, we reported a value of 200 fg injected with a signal-to-noise higher than 5:1 for the same congener [5]. Sensitivity improvement was obtained by an optimisation of the damping gas pressure of helium inside the trap. Moreover, in a recent report published by Hayward et al. [6] on sensitivity specification for 2,3,7,8-TCDD using a quadrupole ion trap in MS/MS mode, the author examined the optimisation of 26 parameters with the aim of setting a benchmark sensitivity for a selected ion trap instrument. An average signal-to-noise ratio of 49:1 for 400 fg of 2,3,7,8-TCDD injected was reached in optimal conditions (i.e. not routine conditions). All the sensitivity tests performed and reported in the literature for TCDD using either an internal or external ionisation system for ion trap generally agree with the criteria that the instrument detection limit (IDL) is approximately 5–10 times higher than the IDL obtained with an HRMS sector instrument. In order to partially fill in the gap of sensitivity, two approaches can be considered: one is to increase the sample intakes; the other one is to inject higher amount into the GC column. The first option is not really in agreement with the requirements of a screening method because a stronger clean-up would be required. The second option is more attractive, keeping in mind that a specific clean-up would still be required for large volume injection.

In this paper, the second option is evaluated in terms of a programmable temperature vaporizer-large volume (PTV-LV) injection coupled to GC/MS/MS for the monitoring of the 17 PCDD/F congeners in foodstuffs at sub-ppt levels. The alternative method was then assessed and compared to the reference method for each congener's mean value by an ANOVA according to a protocol using several matrices at different levels of contamination [7]. A comparison of means values in TEQ is also reported.

2. Experimental

2.1. Chemicals

Toluene, hexane, dichloromethane, pentane, ethyl acetate and water were from Riedel de Haën, Seelze, Germany (Pestanal reagents). Nonane puriss analytical-reagent grade standard for GC was from Fluka, Steinheim, Germany. Sulphuric acid 95–97% and sodium sulphate anhydrous were Baker analysed reagent (J.T. Baker, Deventer, Holland). Silica gel 60 (0.063–200 nm) for column chromatography was from Merck, Darmstadt, Germany. Borosilicate solids glass beads (3 mm) were from Aldrich (Milwaukee, WI, USA). Glass fiber thimbles (43 mm × 123 mm) were from Schleicher and Schuell (Dassel, Germany).

Nitrogen gas was from Air Liquide (Liege, Belgium) and chromatographic pure grade helium gas, 99.9999% was purchased from Air Products (Vilvoorde, Belgium). The internal standard solution of the seventeen 2,3,7,8-chloro-subsituted $^{13}C_{12}$ congeners labelled PCDD/Fs (EDF-4144), the calibration standard solution (EDF-4143) and the syringe standard (EDF-4145) were purchased from Cambridge Isotope Laboratory (Andover, MS, USA). The EDF-4143, EDF-4144 and EDF-4145 concentrations of the natives and labelled congeners are summarised in a previous paper [8].

2.2. Quality control samples and certified reference material

The evaluation of the screening method is based on the simultaneous comparison of means' values obtained for both techniques on different types of samples and levels. Five different matrices have been selected: beef fat, yolk, foetal bovine serum, animal feed and milk powder.

All the samples are quality control (QC). Beef fat, eggs yolk and serum samples were fortified with PCDD/Fs at different levels whereas the animal feed QC was naturally contaminated with dioxins and furans. The last selected matrix was a certified reference material BCR-607 (IRMM, Geel, Belgium), a spray-dried milk powder. The dioxin levels in TEQ for these five matrices spanned two orders of magnitude (i.e. from 0.2 to 25 pg-TEQ g^{-1}). For each matrix, a series of 10 replicates was carried out. Five samples out of the 10 were analysed by the PTV-LV-GC/MS/MS method while the remaining five samples were injected into the GC/HRMS instrument. Thus, a total of 50 analyses have been performed.

2.3. Samples preparation

Ten grams of milk powder was Soxhlet extracted for 16 h using dichloromethane–pentane (1:1). Freeze-dried yolk and 30 g of animal feed samples were extracted by pressurized liquid extraction (PLE) using a Dionex ASE 200 (Sunnyvale, CA, USA). Working conditions are described in details elsewhere [8]. Forty millilitres of serum samples were extracted by solid phase extraction (SPE) using C_{18} non-endcapped cartridges by a protocol reported by Focant and De Pauw [9]. Ten microlitres of the standard solution of 2,3,7,8-chloro substituted labelled dioxins (EDF-4144) was spiked prior to extraction on animal feed and serum samples whereas 3 g of milk powder fat and 4 g of yolk and beef fat samples were spiked after the extraction step. The clean-up was performed on the Power-Prep system (Fluid Management System Inc., Waltham, MA, USA) and details concerning the system have been already published [8,9]. Briefly, high fat content extracts (i.e. milk powder, yolk and beef fat samples) were processed through a set of disposable columns: a high capacity acid silica column, a small multi-layer silica column, a basic alumina column and a PX-21 carbon column. Low fat content extracts (i.e. animal feed and serum samples) were purified in a similar way but the clean-up was simplified by the removal of the preliminary high capacity acid silica column. The final extracts (60 ml of toluene) were then concentrated

to approximately 150 μl using a Turbovap II workstation and transferred to conic vials. The remaining toluene was gently evaporated at room temperature. Half of the extracts were kept in 10 μl of toluene prior to PTV-LV-GC/MS/MS injection. The other half of extracts were concentrated to 4 μl in nonane and 5 μl of syringe standard (EDF-4145) was added prior to GC/HRMS injection.

2.4. Analysis

2.4.1. GC/HRMS

The HRMS experiments were performed on an Autospec Ultima (Micromass, Manchester, United Kingdom). The HRMS was connected by a heated transfer line (275 °C) to a Agilent 6890 Series (Palo Alto, CA, USA) gas chromatograph equipped with a A200SE autosampler (CTC Analytics AG, Zwingen, Zwitzerland). The column was a Rtx-5MS 40 m with an internal diameter 0.18 mm and a stationary phase thickness of 0.18 μm (Restek, Interscience, Belgium). Helium was used as the carrier gas at constant flow rate of 1.2 ml min^{-1}. Two microlitres of the final extract in nonane was injected into a split/splitless injector held at 275 °C in splitless mode. The oven temperature was 140 °C for 1 min, followed by an increase to 200 °C at 52 °C min^{-1}, then increase to 235 °C at 2.9 °C min^{-1} and hold for 10 min, finally increase to 300 °C at 6.9 °C min^{-1} and hold for 6 min. HRMS parameters conditions, quantification and insurance quality control for measurements were described previously [8].

2.4.2. PTV-LV-GC/MS/MS

The MS/MS experiments were performed on a Finnigan PolarisQ ion trap (Austin, TX, USA) held at 250 °C. The ion trap was connected by a heated transfer line (300 °C) to

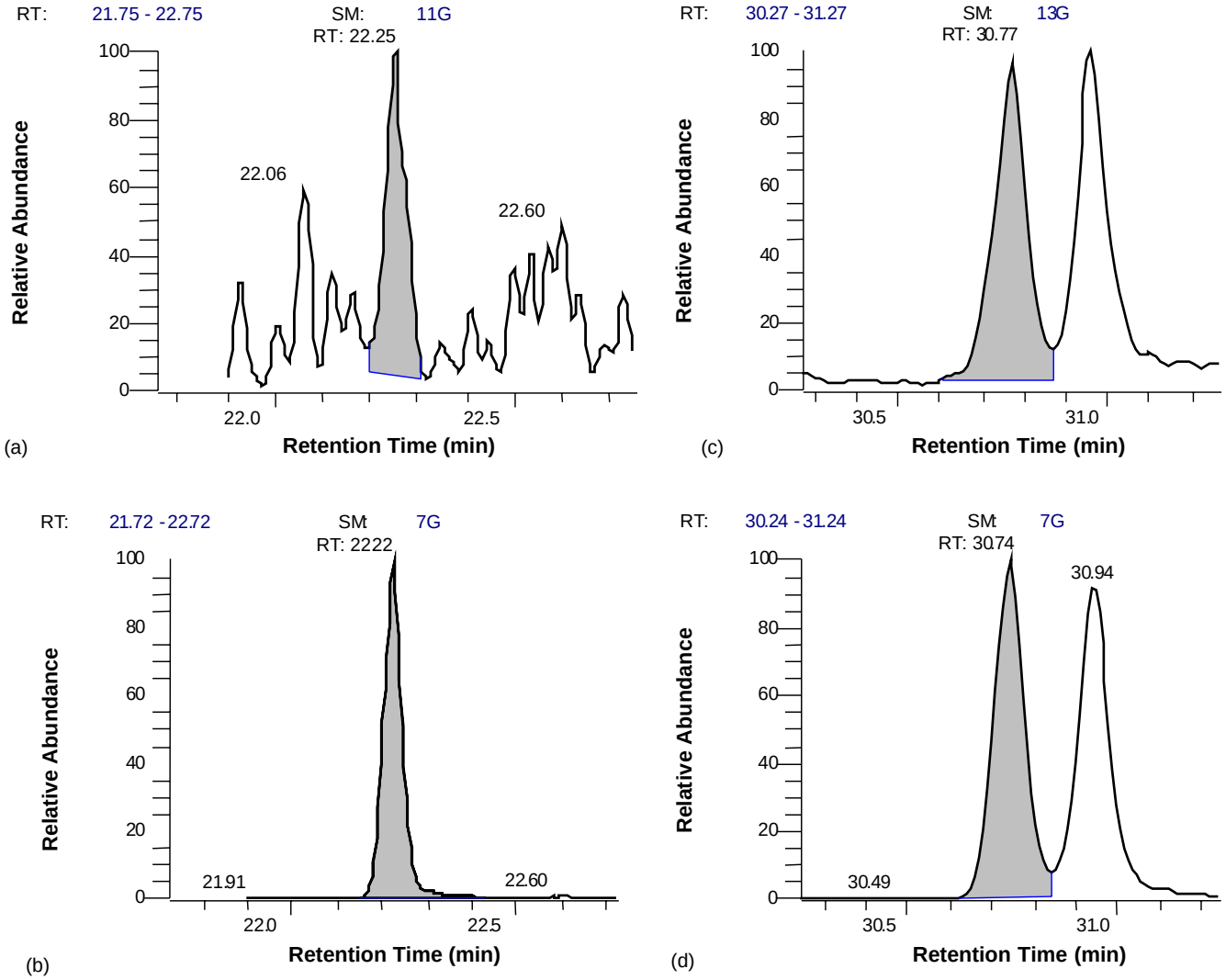

Fig. 1. Typical chromatogram for 2,3,7,8-TCDD (a and b) and for 1,2,3,4,7,8-HxCDF (c and d) from the quality control serum sample. The chromatogram a (c) corresponds to the merged ion current of the sum of the 2,3,7,8-TCDD (1,2,3,4,7,8-HxCDF) native product ions: *m/z* 257 + 259 (*m/z* 309 + 311); the chromatogram b (d) shows the corresponding internal standard $^{13}C_{12}$ 2,3,7,8,-TCDD ($^{13}C_{12}$ 1,2,3,4,7,8-HxCDF) with product ions: *m/z* 268 + 270 (*m/z* 320 + 322).

a Thermoquest Trace GC 2000 (Milan, Italy) gas chromatograph equipped with a Combi Pal autosampler (CTC Analytics AG, Zwingen, Zwitzerland). Xcalibur 1.2 was used for data acquisition. The analyses were carried out using a 40 m × 0.18 mm i.d. × 0.18 μm Rtx-5MS and using He as the carrier gas at constant flow rate of 1.2 ml min^{-1}. The column was directly connected to a BEST PTV injector. The liner used was a Silco Sleeve liner with glass wool from (Restek, Interscience, Belgium). The oven temperature was maintained at 100 °C for 6 min, ramped at 52 °C min^{-1} to 200 °C; ramped at 2.9 °C min^{-1} to 250 °C for 6 min; ramped at 2.9 °C min^{-1} to 260 °C and finally ramped at 10 °C min^{-1} to 300 °C for 5 min.

2.5. Identification and quantification by MS/MS

Only 2,3,7,8-chloro-substituted congeners were followed. Peaks were detected when a signal-to-noise ratio (S:N) is ≥3. The native peak maxima should fall within 3 s of their corresponding ^{13}C labelled analogues. Fig. 1 shows chromatograms for 2,3,7,8-TCDD (a and b) and for 1,2,3,4,7,8-HxCDF (c and d) obtained from the serum quality control sample by PTV-LV-GC/MS/MS. For both congeners, the upper chromatograms (a and c) represent the native daughter ions signal; the lower chromatograms (b and d) represent their corresponding ^{13}C labelled standard daughter ions signal. Chromatographic requirements were the same as for HRMS analysis as described in the EPA 1613 method revision B [10] or more recently in the Directives 2002/69/EC or 2002/70/EC [11,12].

Quantification for tandem mass spectrometry was also by isotopic dilution technique, but applied on product ions instead of molecular ions. The sum of the two most abundant product ions for native and ^{13}C labelled standard were used to reconstruct the ion current as shown in Fig. 1.

Calibration was carried out by injecting a five-points calibration solution (EDF-4143) by PTV-LV-GC/MS/MS. The linear response range (i.e. in the working range) as well as the relative response factor (RRF) associated to each congener were established. The RRF value was calculated as:

$$\mathrm{RRF} = \frac{(A^1_{\mathrm{native},i} + A^2_{\mathrm{native},i})C_\mathrm{s}}{(A^1_{\mathrm{std},i} + A^2_{\mathrm{std},i})C_\mathrm{n}} \quad (1)$$

where A^1_{native} and A^2_{native} are the areas of the primary and secondary daughter ion of the native congener; A^1_{std} and A^2_{std} are the areas of the primary and secondary daughter ion of the internal ^{13}C standard; C_n is the concentration of the native compound in the calibration solution and C_s is the concentration of the labelled compound in the calibration solution.

When the concentration ratio of native to analogue standard ^{13}C was plotted against the area ratio of native to analogue standard ^{13}C, the slope of the curve gives the corresponding RRF value. Good linearity was achieved and regression lines were characterized by correlation coefficients (R^2) higher than 0.99 for all the congeners. Table 1 gives an overview of the average RRFs and their corresponding coefficients of variation (CVs) calculated during a calibration of the 17 PCDD/F congeners. The values are compared to the results obtained during a classical GC/HRMS calibration exercise. As can be seen, CVs in the range of 2.3 and 21% were obtained by the alternative method. CVs are generally higher than those obtained by the reference HRMS method but they are still acceptable.

Table 1
Average RRFs and their corresponding CVs for individual congener calibrations by PTV-LV-GC/MS/MS and GC/HRMS

Compounds	MS/MS RRF	CV (%)	HRMS RRF	CV (%)
2,3,7,8-TCDD	1.01	2.3	1.02	7.0
1,2,3,7,8-PeCDD	0.86	10.9	0.92	9.7
1,2,3,4,7,8-HxCDD	1.08	9.2	1.09	6.1
1,2,3,6,7,8-HxCDD	0.79	15.6	0.88	4.8
1,2,3,7,8,9-HxCDD	0.92	8.5	1.00	5.2
1,2,3,4,6,7,8-HpCDD	1.09	7.0	0.91	6.0
OCDD	0.99	3.0	1.04	3.1
2,3,7,8-TCDF	0.87	9.2	0.94	2.5
1,2,3,7,8-PeCDF	0.84	4.3	0.84	6.8
2,3,4,7,8-PeCDF	0.82	13.7	0.98	3.8
1,2,3,4,7,8-HxCDF	1.11	5.8	1.03	5.7
1,2,3,6,7,8-HxCDF	1.08	11.4	0.94	3.4
1,2,3,7,8,9-HxCDF	0.98	7.0	0.94	7.3
2,3,4,6,7,8-HxCDF	1.00	15.4	1.02	6.7
1,2,3,4,6,7,8-HpCDF	1.02	4.7	1.05	4.1
1,2,3,4,7,8,9-HpCDF	1.10	16.5	0.75	10.0
OCDF	1.09	21.0	1.07	8.2

The individual 2,3,7,8-PCDD/F congener quantification is then calculated as follows:

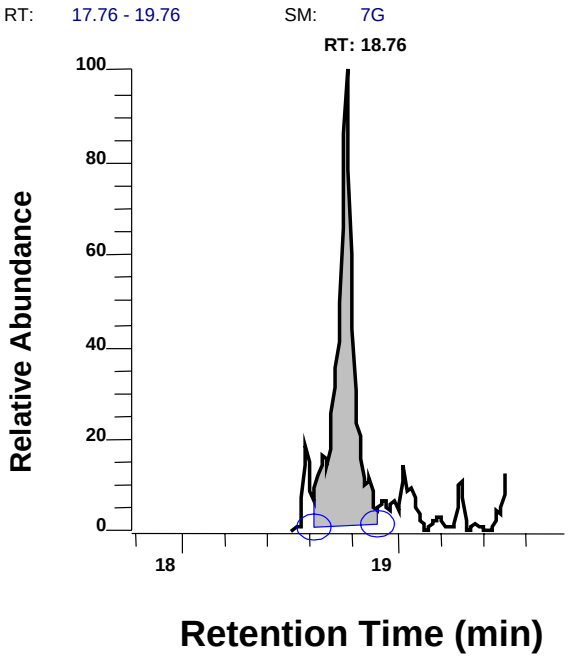

Fig. 2. Instrument detection limit of 200 fg 2,3,7,8-TCDD (*m*/*z* 257 + 259) with S:N ratio ≥ 5:1 by PTV-LV-GC/MS/MS.

$$[\text{congener}]_i = \frac{(A^1_{\text{native},i} + A^2_{\text{native},i})Q_i}{(A^1_{\text{std},i} + A^2_{\text{std},i})\text{RRF}_i m} \quad (2)$$

where $[\text{congener}]_i$ is the concentration of the congener i (ng kg^{-1}); areas were defined above (Eq. (1)); Q_i is the amount of the corresponding internal standard i spiked (ng) in the sample; RRF_i is the relative response factor of the congener i and m is the weight of the sample (kg). Finally, the quantification in TEQ is calculated using the toxic equivalence factors (TEFs) reported by the World Health Organisation (1998) [13].

3. Results and discussion

3.1. MS/MS optimisation

Development of dioxin analysis methods based on quadrupole ion trap operated in tandem mode have been reviewed in the literature [14–18]. In the system used for this paper, ions are formed in an external ion source by electron ionisation (EI) mode. They are then accelerated through lenses and introduced into the ion trap analyser. The RF voltage applied to the ring electrode is set to a value that is optimised for optimum trapping efficiency. A buffer gas of helium is continually introduced into the ion trap to slow the motion of ions for better trapping efficiency. Sensitivity is a key issue to provide reliable dioxin results in food and feed at ultra trace levels. In the ion trap, the sensitivity is limited by the trapping efficiency of the precursor ion. To improve the trapping efficiency of the precursor ion, a new hardware that increases the helium damping gas pressure inside the ion trap was installed and evaluated on the instrument. The helium damping gas flow rate inside the trap was preset to a default value of 0.3 ml min^{-1}. By increasing the helium pressure to a flow rate of approximately 1.7 ml min^{-1} (i.e. the optimum), the trapping efficiency of the precursor ion was enhanced; hence increasing the product ion yield. Thus, the IDL in terms of signal-to-noise ratio was increased by roughly a factor 3 for 2,3,7,8-TCDD in MS/MS mode.

Table 2
Main parameters optimised for MS/MS analysis of dioxins and furans

Segments	Compounds	Molecular or precursor ions	CID (V)	Collision time (ms)	q-value	Product ions
1	2,3,7,8-TCDF	306 ($M+2$)	5.5	30	0.45	241/243
	2,3,7,8-TCDF $^{13}C_{12}$	318 ($M+2$)	5.5	30	0.45	252/254
2	2,3,7,8-TCDD	322 ($M+2$)	5	30	0.45	257/259
	2,3,7,8-TCDD $^{13}C_{12}$	334 ($M+2$)	5	30	0.45	268/270
3	1,2,3,7,8-PeCDF	340 ($M+2$)	6	30	0.45	275/277
	1,2,3,7,8-PeCDF $^{13}C_{12}$	352 ($M+2$)	6	30	0.45	286/288
	2,3,4,7,8-PeCDF	340 ($M+2$)	6	30	0.45	275/277
	2,3,4,7,8-PeCDF $^{13}C_{12}$	352 ($M+2$)	6	30	0.45	286/288
	1,2,3,7,8-PeCDD	356 ($M+2$)	6	30	0.45	291/293
	1,2,3,7,8-PeCDD $^{13}C_{12}$	368 ($M+2$)	6	30	0.45	302/304
4	1,2,3,4,7,8-HxCDF	374 ($M+2$)	6	30	0.45	309/311
	1,2,3,4,7,8-HxCDF $^{13}C_{12}$	386 ($M+2$)	6	30	0.45	320/322
	1,2,3,6,7,8-HxCDF	374 ($M+2$)	6	30	0.45	309/311
	1,2,3,6,7,8-HxCDF $^{13}C_{12}$	386 ($M+2$)	6	30	0.45	320/322
	2,3,4,6,7,8-HxCDF	374 ($M+2$)	6	30	0.45	309/311
	2,3,4,6,7,8-HxCDF $^{13}C_{12}$	386 ($M+2$)	6	30	0.45	320/322
	1,2,3,4,7,8-HxCDD	390 ($M+2$)	6	30	0.45	325/327
	1,2,3,4,7,8-HxCDD $^{13}C_{12}$	402 ($M+2$)	6	30	0.45	336/338
	1,2,3,6,7,8-HxCDD	390 ($M+2$)	6	30	0.45	325/327
	1,2,3,6,7,8-HxCDD $^{13}C_{12}$	402 ($M+2$)	6	30	0.45	336/338
	1,2,3,7,8,9-HxCDD	390 ($M+2$)	6	30	0.45	325/327
	1,2,3,7,8,9-HxCDD $^{113}C_{12}$	402 ($M+2$)	6	30	0.45	336/338
	1,2,3,7,8,9-HxCDF	374 ($M+2$)	6	30	0.45	309/311
	1,2,3,7,8,9-HxCDF $^{13}C_{12}$	386 ($M+2$)	6	30	0.45	320/322
5	1,2,3,4,6,7,8-HpCDF	410 ($M+4$)	6	30	0.45	345/347
	1,2,3,4,6,7,8-HpCDF $^{13}C_{12}$	422 ($M+4$)	6	30	0.45	356/358
	1,2,3,4,7,8,9-HpCDF	426 ($M+4$)	6	30	0.45	361/363
	1,2,3,4,7,8,9-HpCDF $^{13}C_{12}$	438 ($M+4$)	6	30	0.45	372/374
	1,2,3,4,6,7,8-HpCDD	410 ($M+4$)	6	30	0.45	345/347
	1,2,3,4,6,7,8-HpCDD $^{13}C_{12}$	422 ($M+4$)	6	30	0.45	356/358
6	OCDD	460 ($M+4$)	6	30	0.45	395/397
	OCDD $^{13}C_{12}$	472 ($M+4$)	6	30	0.45	406/408
	OCDF	444 ($M+4$)	6	30	0.45	379/381
	OCDF $^{13}C_{12}$	456 ($M+4$)	6	30	0.45	390/392

Congeners classification correspond to the elution order on Rtx5-MS 40 m column.

Fig. 2 shows the chromatogram for 200 fg of 2,3,7,8-TCDD injected. The chromatogram is rebuilt with the merged ion current of the two product ions (m/z 257 + 259). Helium also operates as a collision gas during the collision-induced dissociation (CID) process which causes the fragmentation of precursor ions in product ions or daughter ions. Due to the high helium damping gas pressure inside the trap, precursor ion motion is damped and it takes more energy to excite the ions for CID fragmentation. Radio frequency (rf) voltages between 5 and 6 V were applied to the endcap electrodes to fragment tetra-through octa-chlorinated dioxins and furans (as reported in Table 2). The product ions masses, characterized by a loss of $COCl^{\cdot}$, are then scanned by ramping a resonance ejection rf voltage that sequentially eject them from low to high m/z.

Practically, after retention time determination by full scan acquisition for all the congeners, an acquisition function of the mass spectrometer was built by slicing the chromatogram into six different segments. Each segment correspond to a group of chlorination, excepted for TCDD and TCDF for which their retention time allowed to build separate acquisition tables. For PCDD/Fs analysis in MS/MS mode, the trapping parameter q_z was set to an optimum value of 0.45 [19,20]. To optimise CID voltages, several injections of one EDF-4143 calibration point standard solution were needed. An acquisition table was built (see Table 2) summarising the optimised MS/MS parameters.

3.2. PTV-LV injection for PCDD/Fs analysis

Large volume injection into a PTV injector can be done in different modes: 'at once', by a speed control injection, or using multiple injection [21]. The first two modes of injection are normally preferred with the type of PTV used here. 'At once', the sample is introduced at a relatively high speed (e.g. 10 μl s^{-1}) whereas the speed control injection mode introduces the sample at a rate that is theoretically equal to the evaporation rate. Both modes have been tested and best results were obtained by injecting the sample at a speed control of 2.1 μl s^{-1}.

Toluene was used to optimise the PTV parameters. This solvent was selected because PCDD/Fs are collected from the last clean-up step in toluene. It is characterized by a high boiling point (i.e. 110 °C) and is therefore not the easiest solvent to use for large volume injection. PTV injection is divided into four phases: the injection, the vaporization, the transfer and finally the cleaning phase. During the injection, the split valve is open (solvent split injection mode) and the sample is introduced into the cold liner with glass wool set at a temperature below the boiling point of toluene (100 °C). PCDD/Fs are characterized by high boiling points and no significant losses occurred at 100 °C. During the evaporation step, the PTV temperature is raised to 120 °C for 30 s in order to eliminate the solvent. The solvent is vented through the split valve at an optimised split flow of 100 ml min^{-1}. Once the solvent eliminated, the third step consists in transferring the components to the analytical column. The split valve is closed and the temperature rapidly raised to 300 °C in splitless mode for 1 min. After transfer of the components, the split valve is opened again (100 ml min^{-1}) and the liner is kept at 300 °C during the GC run for cleaning.

First trials were performed with 30 μl of toluene. Toluene is difficult to pump out; it remains in the GC/MS system for a minimum of 2 h after injection. An alternative option to solve the problem was to equip the system with a back-flush device for PTV injection. If considerable improvements for toluene were noticed, unfortunately significant losses of components were also observed. The reason is mostly due to the use of a high boiling point solvent. Back-flush applications for PTV injection seem to be suitable with lower boiling point solvent (e.g. pentane, hexane).

The problem was finally partially solved by evaporating the final extract to a lower volume and injecting 10 μl into the PTV injector.

3.3. Statistical evaluation

The validation consisted in comparing the PTV-LV-GC/MS/MS alternative method to the GC/HRMS method on different types of samples. Five different matrices (i.e. beef fat, yolk, milk powder, animal feed and serum) were selected for this study. Matrices were chosen to cover commonly encountered interferences. The sample intakes and the sample preparation were similar for both analytical techniques. Furthermore, five replicates per method were carried out. An overview of the mean values with their corresponding standard deviations (S.D.) is presented in Table 3. Results were expressed in parts-per-trillion (ng kg^{-1}) for each individual congener and also in WHO-TEQ for the sum of the 17 congeners. As can be seen, the PCDD/Fs levels in TEQ vary from 0.2 to 25 ng WHO-TEQ kg^{-1}. Good agreement between the mean values in TEQ was found, even if higher standard deviations for the screening method were obtained. This remark was also true for individual congener. At this stage, if we conduct a comparison between means per congener and also per matrix, 74 tests of means comparison should be necessary (see Table 3). In order to be able to draw global conclusions, statistical treatment of data using an analysis of variance allows a simultaneous comparison of means [22]. For multi-factor experimental designs, ANOVA can provide separate variance estimates for each factors.

The general equation used here for the ANOVA is the following:

$$SCE_t = SCE_r + SCE_a + SCE_b + SCE_{ab} \quad (4)$$

where SCE_t is the sum of the square of the total deviations; SCE_r is the sum of the square of the residual deviations; SCE_a is the sum of the square of the deviations due to the method factor; SCE_b is the sum of the square of the deviations due to the matrix factor, SCE_{ab} is the sum of the square of the deviations due to the interaction between method and

Table 3
PCDD/Fs mean values expressed in parts-per-trillion (ng kg^{-1}) and their corresponding standard deviations for PTV-LV-GC/MS/MS and GC/HRMS methods

Dioxins/furans	Beef fat				Yolk				Milk powder BCR 607				Assigned value (ng kg^{-1})
	MS/MS		HRMS		MS/MS		HRMS		MS/MS		HRMS		
	Mean (ng kg^{-1})	S.D. ($n = 5$)	Mean (ng kg^{-1})	S.D. ($n = 5$)	Mean (ng kg^{-1})	S.D. ($n = 5$)	Mean (ng kg^{-1})	S.D. ($n = 5$)	Mean (ng kg^{-1})	S.D. ($n = 5$)	Mean (ng kg^{-1})	S.D. ($n = 5$)	
2,3,7,8-TCDD	0.28	0.06	0.38	0.04	2.07	0.42	2.37	0.18	0.24	0.03	0.29	0.01	0.25
1,2,3,7,8-PeCDD	2.04	0.32	1.71	0.11	9.79	1.87	8.68	0.35	0.78	0.12	0.81	0.06	0.79
1,2,3,4,7,8-HxCDD	2.04	0.14	1.82	0.17	7.63	0.54	9.36	0.39	0.45	0.18	0.46	0.04	0.42
1,2,3,6,7,8-HxCDD	2.07	0.27	2.25	0.11	10.65	2.55	10.64	0.09	1.02	0.07	1.09	0.09	0.98
1,2,3,7,8,9-HxCDD	1.81	0.21	1.95	0.04	7.96	1.41	9.56	0.31	0.30	0.25	0.38	0.03	0.34
1,2,3,4,6,7,8-HpCDD	2.02	0.31	1.50	0.19	9.18	1.05	6.19	0.09	nd		nd		Not assigned
OCDD	4.60	1.66	4.42	1.35	24.61	1.07	21.76	1.56	nd		nd		Not assigned
2,3,7,8-TCDF	2.50	0.82	0.34	0.15	2.95	0.52	2.91	0.11	0.08	0.03	nd		0.05
1,2,3,7,8-PeCDF	1.99	0.20	2.23	0.06	11.36	0.77	12.27	0.03	0.08	0.03	nd		0.05
2,3,4,7,8-PeCDF	1.69	0.71	2.12	0.04	11.52	0.96	12.38	0.26	1.81	0.20	1.81	0.09	1.81
1,2,3,4,7,8-HxCDF	1.94	0.40	1.91	0.11	9.40	2.08	9.63	0.06	0.84	0.08	0.92	0.04	0.94
1,2,3,6,7,8-HxCDF	2.14	0.17	1.95	0.09	9.75	1.61	10.89	0.48	1.03	0.15	1.08	0.04	1.01
1,2,3,7,8,9-HxCDF	2.28	0.58	2.21	0.17	10.55	1.26	11.14	0.27	nd		nd		Not assigned
2,3,4,6,7,8-HxCDF	1.97	0.37	1.93	0.12	10.63	1.37	10.69	0.35	1.03	0.12	1.07	0.08	1.07
1,2,3,4,6,7,8-HpCDF	1.89	0.52	2.14	0.09	8.91	2.30	11.34	0.57	nd		nd		Not assigned
1,2,3,4,7,8,9-HpCDF	1.65	0.28	2.14	0.13	9.02	0.79	10.21	0.43	nd		nd		Not assigned
OCDF	5.14	0.87	3.57	0.23	18.65	1.57	17.85	0.30	nd		nd		Not assigned
WHO-TEQ-PCDD/F	5.10	0.32	4.72	0.22	25.42	2.36	25.61	0.23	2.24	0.38	2.50	0.08	2.43

Dioxins/furans	Animal feed				Bovine serum			
	MS/MS		HRMS		MS/MS		HRMS	
	Mean (ng kg^{-1})	S.D. ($n = 5$)	Mean (ng kg^{-1})	S.D. ($n = 5$)	Mean (ng kg^{-1})	S.D. ($n = 5$)	Mean (ng kg^{-1})	S.D. ($n = 5$)
2,3,7,8-TCDD	nd		nd		0.02	0.00	0.02	0.00
1,2,3,7,8-PeCDD	0.08	0.06	0.02	0.02	0.10	0.02	0.08	0.01
1,2,3,4,7,8-HxCDD	0.29	0.19	0.14	0.04	0.09	0.01	0.07	0.02
1,2,3,6,7,8-HxCDD	2.38	0.44	2.27	0.14	0.11	0.02	0.10	0.01
1,2,3,7,8,9-HxCDD	0.76	0.16	0.84	0.12	0.12	0.03	0.08	0.01
1,2,3,4,6,7,8-HpCDD	144.2	6.99	166.4	12.18	0.09	0.02	0.11	0.02
OCDD	962.7	158.1	927.7	124.1	0.19	0.05	0.21	0.01
2,3,7,8-TCDF	0.11	0.06	0.11	0.03	0.09	0.03	0.03	0.00
1,2,3,7,8-PeCDF	0.04	0.04	0.02	0.01	0.12	0.01	0.12	0.01
2,3,4,7,8-PeCDF	0.09	0.10	0.06	0.02	0.11	0.01	0.11	0.01
1,2,3,4,7,8-HxCDF	0.14	0.10	0.08	0.01	0.10	0.02	0.09	0.00
1,2,3,6,7,8-HxCDF	0.04	0.04	0.05	0.01	0.04	0.01	0.10	0.01
1,2,3,7,8,9-HxCDF	nd		nd		0.10	0.02	0.10	0.01
2,3,4,6,7,8-HxCDF	nd		0.04	0.02	0.08	0.01	0.10	0.01
1,2,3,4,6,7,8-HpCDF	1.93	0.32	1.70	0.28	0.13	0.01	0.09	0.00
1,2,3,4,7,8,9-HpCDF	0.24	0.23	0.21	0.06	0.08	0.02	0.09	0.01
OCDF	11.46	0.78	13.55	1.54	0.13	0.03	0.15	0.03
WHO-TEQ-PCDD/F	2.15	0.13	2.20	0.23	0.26	0.03	0.23	0.02

The sum in WHO-TEQ is also reported.

matrix. The ANOVA can also take into account possible interactions effects (e.g. between method and matrix).

In our example, several types of means were calculated: means per sample, means per matrix or means per method. Thus, to test the comparison between means, hypotheses tests are interesting statistical tools to see if our claims were correct. These tests were developed to facilitate the decision-making at a significance level. The first step was to specify the null hypothesis (H_0). The purpose of this work was to assume the equality between means:

$$H_0 : \mu_1 = \mu_2 = \ldots = \mu_n \quad (3)$$

The alternative hypothesis (H_1) would be:

$$H_1 : \mu_1 \neq \mu_2 \neq \ldots \neq \mu_n \quad (4)$$

The second step was to select a significance level for rejection of H_0. A typical level of $\alpha = 0.05$ was chosen. As mentioned above, the ANOVA can be used to test the comparison between multiple means. In addition, it can provide separate variance estimates for each parameter. The comparison between two variances was done by the statistical test of Fisher. In the third step, the statistic test value (i.e. the calculated *F*-value) was then used to decide whether or not the null hypothesis should be rejected in our hypothesis test at the significance level. This was done by comparing the calculated *F*-value to its critical tabulated *F*-value. In our validation study, three different *F*-values were calculated per congener: F_{method}, F_{matrix} and $F_{interaction}$. Fisher test values were calculated by dividing the parameter variance (i.e. method, matrix or interaction) by the residual variance.

In statistical hypothesis test, the probability value (*P*-value) is often used. Small *P*-values suggest that the null hypothesis is unlikely to be true. If *P*-values are smaller than the pre-established significance level then the null hypothesis is rejected, suggesting that the alternative hypothesis may be true.

ANOVA results are presented in Table 4. For each congener, the calculated *F*-values, their corresponding critical tabulated *F*-values and the *P*-values are reported. The interpretation of the results indicates that F_{method} is always below its critical *F*-value. The method effect is therefore not significant for the 17 PCDD/Fs. The null hypothesis is not rejected and this is confirmed by high probability *P*-values. Nevertheless, three congeners (2,3,7,8-TCDF; 1,2,3,4,7,8-HxCDD and 1,2,3,4,6,7,8-HpCDD) have *P*-values below 0.5. indicating that there is only less than 0.5 chance that H_0 is true. On the other hand, the matrix effect is significant for all the congeners; H_0 is rejected. As dioxin and furan levels in the different matrices were covering sometimes several orders of magnitude, the rejection of H_0 is obvious. Besides, the interaction between method and matrix pointed out that these effects are pure (interaction is not significant) for most of the 2,3,7,8 congeners. H_0 is not rejected and it brings out the good selectivity of the alternative method for most of them. However, the three congeners (mentioned above) have *P*-values smaller than the significance level ($P < 0.05$). It indicates the influence of specific matrix on the mean values for the comparison between both techniques. For instance, 2,3,7,8-TCDF mean value in beef fat is greatly higher by PTV-LV-GC/MS/MS analysis than by GC/HRMS analysis (see Table 3). It might be coming from a specific matrix interference.

Another possibility to interpret the raw data in the comparison between two methods, is the research of a functional relationship between the results [22]. Establishing a linear relationship between the alternative method compared to the reference method enables to detect bias. If the

Table 4
Synthesis of ANOVA results using Fisher test for the 17 PCDD/F congeners

Dioxins/furans	Calculated *F*-values			Tabulated *F*-values at the 95% significance level			*P*-values ($\alpha = 0.05$)		
	Method	Matrix	Interaction	Method	Matrix	Interaction	Method	Matrix	Interaction
2,3,7,8-TCDD	0.2346	38.77	0.313	4.17	2.92	2.92	0.6314	<0.05	0.8158
1,2,3,7,8-PeCDD	0.0918	30.98	0.228	4.08	2.61	2.61	0.7640	<0.05	0.9205
1,2,3,4,7,8-HxCDD	0.6845	232.09	6.117	4.08	2.61	2.61	0.4138	<0.05	<0.05
1,2,3,6,7,8-HxCDD	0.0001	23.39	0.007	4.08	2.61	2.61	0.9921	<0.05	0.9999
1,2,3,7,8,9-HxCDD	0.2412	53.93	1.015	4.08	2.61	2.61	0.6263	<0.05	0.4126
1,2,3,4,6,7,8-HpCDD	0.6011	316.16	3.779	4.17	2.92	2.92	0.4442	<0.05	<0.05
OCDD	0.0085	43.39	0.029	4.17	2.92	2.92	0.9271	<0.05	0.9932
2,3,7,8-TCDF	1.3020	15.04	4.638	4.17	2.92	2.92	0.2623	<0.05	<0.05
1,2,3,7,8-PeCDF	0.4106	336.58	1.018	4.17	2.92	2.92	0.5269	<0.05	0.3995
2,3,4,7,8-PeCDF	0.0029	5.85	0.018	4.08	2.61	2.61	0.9574	<0.05	0.9993
1,2,3,4,7,8-HxCDF	0.0004	7.57	0.003	4.08	2.61	2.61	0.9841	<0.05	0.9999
1,2,3,6,7,8-HxCDF	0.0102	8.59	0.063	4.08	2.61	2.61	0.9201	<0.05	0.9924
1,2,3,7,8,9-HxCDF	0.0029	6.56	0.013	4.35	3.49	3.49	0.9575	<0.05	0.9871
2,3,4,6,7,8-HxCDF	0.0001	7.15	0.000	4.17	2.92	2.92	0.9921	<0.05	0.9999
1,2,3,4,6,7,8-HpCDF	0.0566	6.11	0.231	4.17	2.92	2.92	0.8136	<0.05	0.8741
1,2,3,4,7,8,9-HpCDF	0.0354	7.29	0.059	4.17	2.92	2.92	0.8520	<0.05	0.9808
OCDF	0.0002	5.34	0.102	4.17	2.92	2.92	0.9888	<0.05	0.9582
PCDD/Fs WHO-TEQ	8E−06	6.51	0.0014	4.08	2.61	2.61	1.0000	<0.05	1.0000

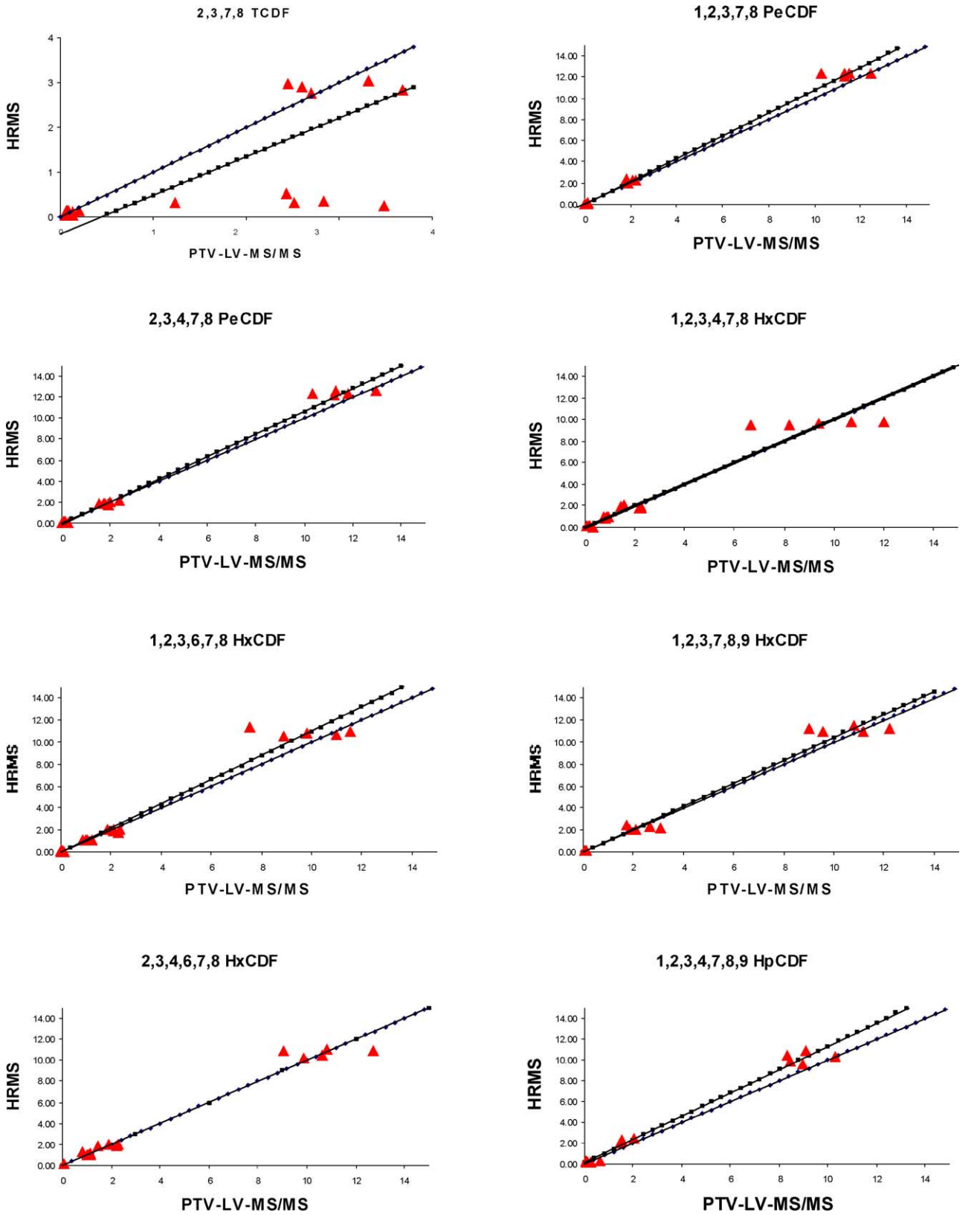

Fig. 3. Representation of the functional relationships between the PTV-LV-GC/MS/MS method and the GC/HRMS method for the PCDD/F congeners.

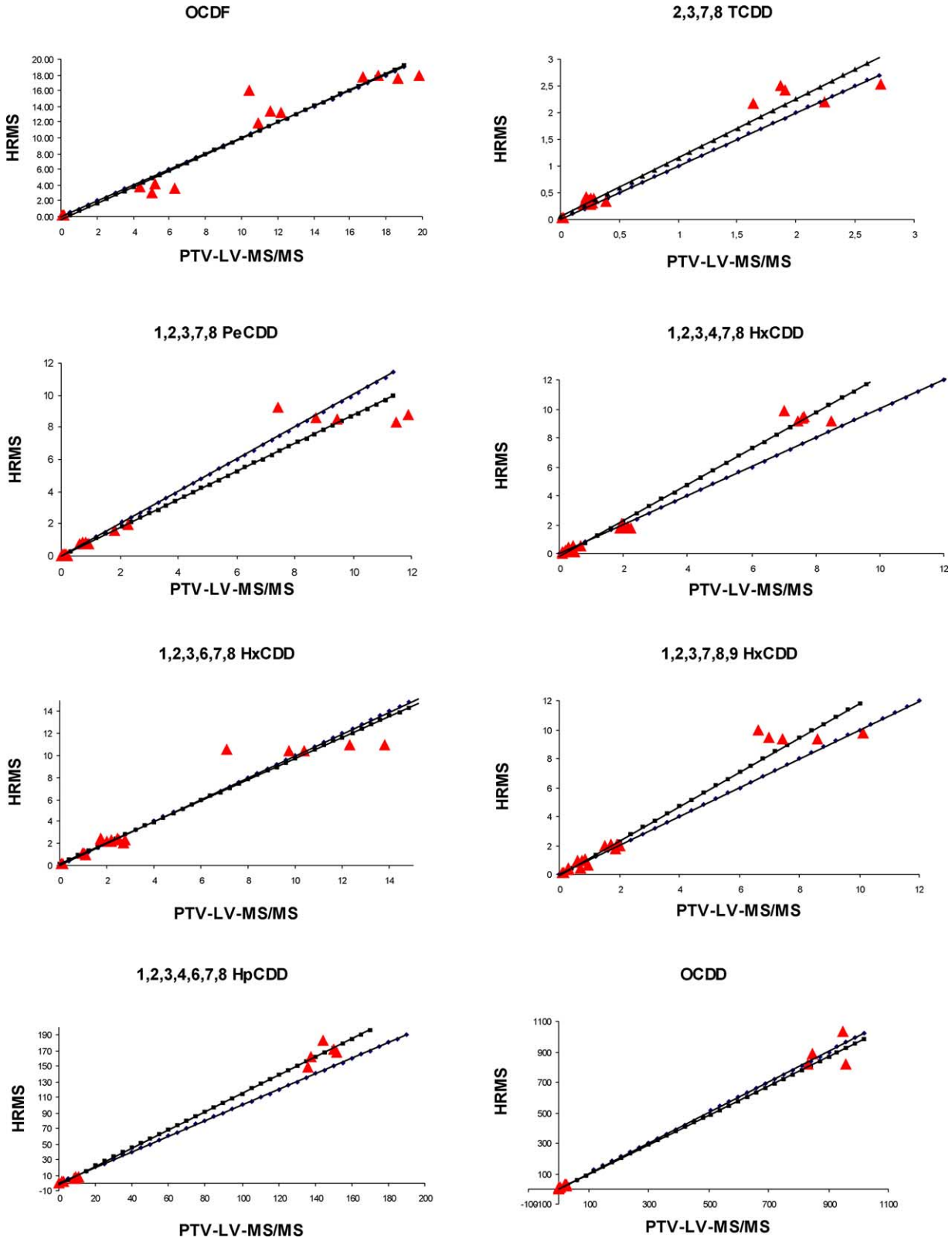

Fig. 3. (*Continued*).

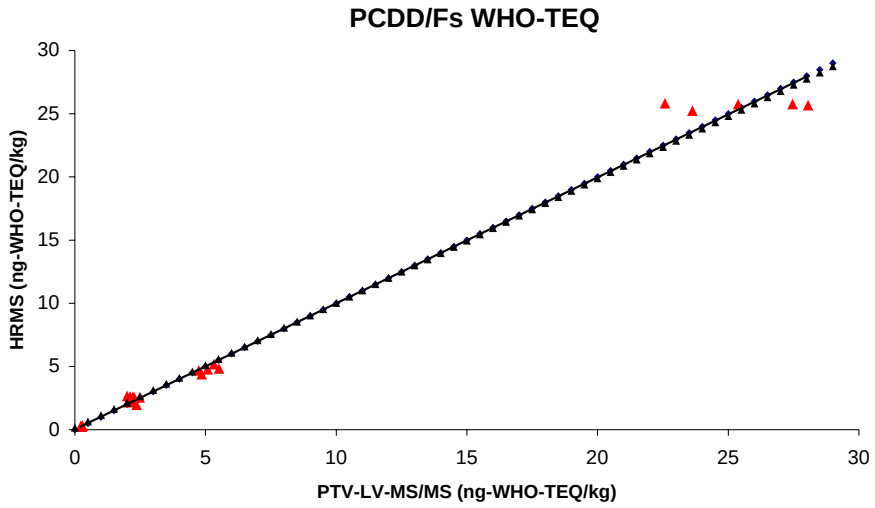

Fig. 4. Representation of the functional relationships between the PTV-LV-GC/MS/MS method and the GC/HRMS method in WHO-TEQ.

functional relationship is not merged with the bisecting line, two types of bias can be detected: a systematic bias or a bias per rotation. The first case is characterized by a constant bias between the methods; the second one is proportional to the concentration level. Fig. 3 shows the functional relationship between PTV-LV-GC/MS/MS method and GC/HRMS method for most of the 2,3,7,8 congeners. Two regression curves were plotted: the bisecting line and the functional relationship between the methods calculated by the least rectangular regression method. For the congener detected in the five matrices by both techniques, 25 raw data points are also represented on the graphs by a triangular shape. The statistical conclusions drawn for the three congeners characterized by low interaction *P*-values between method and matrix is graphically highlighted in Fig. 3. A systematic bias is observed for the 2,3,7,8-TCDF congener whereas a rotation bias increasing with levels is noticed for 1,2,3,4,7,8-HxCDD and 1,2,3,4,6,7,8-HpCDD. Regarding the others congeners, some are practically merged with the bisecting line while others are characterized by a not significant bias.

In TEQ, the results indicate that, in the range of 0.2–25 ng WHO-TEQ kg^{-1} using five different matrices, no bias between the methods was observed as it can be seen in Fig. 4.

4. Conclusions

The development of an alternative PTV-LV-GC/MS/MS method for dioxin levels monitoring in food has been carried out. The statistical evaluation performed on five different matrices indicates that the screening method is suitable for dioxin monitoring at ppt level in foodstuffs. Even if discrepancies were observed for three PCDD/F congeners, the results clearly showed, in TEQ, the good agreement between both methods. It fulfils the European Directives [11,12] analytical requirements regarding screening approaches. Consequently, PTV-LV-GC/MS/MS is an attractive technique and can be used as a cost effective complementary method to HRMS for dioxin levels monitoring in food and feed.

However, the capacity of the method as a high sample throughput technique was not assessed in this paper. There is not been enough time yet to conclude that the method can deal with hundreds or thousands samples per year. The drawback of the technique, as all mass spectrometry techniques, is the maintenance of the system to be used at its maximum performances capacity to detect low ppt levels. This is obviously the key issue for dioxin monitoring.

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