High-throughput biomonitoring of dioxins and polychlorinated biphenyls at the sub-picogram level in human serum

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Abstract

We report on the use of a state-of-the-art method for the measurement of selected polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and polychlorinated biphenyls in human serum specimens. The sample preparation procedure is based on manual small size solid-phase extraction (SPE) followed by automated clean-up and fractionation using multi-sorbent liquid chromatography columns. SPE cartridges and all clean-up columns are disposable. Samples are processed in batches of 20 units, including one blank control (BC) sample and one quality control (QC) sample. The analytical measurement is performed using gas chromatography coupled to isotope dilution high-resolution mass spectrometry. The sample throughput corresponds to one series of 20 samples per day, from sample reception to data quality cross-check and reporting, once the procedure has been started and series of samples keep being produced. Four analysts are required to ensure proper performances of the procedure. The entire procedure has been validated under International Organization for Standardization (ISO) 17025 criteria and further tested over more than 1500 unknown samples during various epidemiological studies. The method is further discussed in terms of reproducibility, efficiency and long-term stability regarding the 35 target analytes. Data related to quality control and limit of quantification (LOQ) calculations are also presented and discussed.

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Keywords: Solid-phase extraction; Isotope dilution; High-resolution mass spectrometry; Human serum; Biomonitoring; Polychlorinated dibenzo-p-dioxins; Polychlorinated dibenzofurans; Polychlorinated biphenyls; Limit of quantification; Multi-level quality control

1. Introduction

Human biomonitoring consists in the assessment of the internal dose exposure of individuals by measuring a toxicant level in body matrices. In the case of lipophilic compounds like polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs), relevant matrices are adipose tissues, blood and milk. Those matrices can nearly be used indifferently as far as analyte levels are reported on a lipid weight basis because equilibration of those toxicants takes place between the lipids of adipose tissue, blood and milk [1,2]. In other words, levels measured in one of those matrices reflect what would also be measured in the other two. Because adipose tissue collection is a rather invasive procedure requiring surgery, it is usually avoided to ease patient volunteering in epidemiological studies. Breast milk collection is a much less invasive procedure but has the double disadvantage to be limited to a specific part of the general population and to require great care concerning the time point at which samples are collected in regards of toxicant depuration while breast feeding is taking place. Human milk monitoring is thus more valuable as part of a food control strategy to protect the young consumers from excessive toxicant intakes. Blood then appears as the preferred matrix as it can be relatively easily obtained by intravenous withdrawing from any individual without previously cited inconvenients. Considering blood for analysis however has the disadvantage to lower the target compound levels as the lipid content is below 1% in weight. Enrichment procedures followed by ultra-sensitive detection devices are therefore required to ensure proper detectability and measurements.

Gas chromatography (GC) coupled to 13C-based isotope dilution (ID) high-resolution (HR) mass spectrometry (MS) is the hyphenated instrumentation of choice to measure ultra-trace levels [detection limits at the part per trillion (ppt) level] of PCDDs, PCDFs and PCBs [3]. Analytical measurement methods based on the use of GC–IDHRMS can easily isolate and quantify...
the target toxic compounds, namely, the 2,3,7,8-substituted PCDD/F congeners as well as the non-ortho (NO) and mono-ortho-PCB (MO-PCB) congeners that have been assigned a toxic equivalent factor (TEF) by the World Health Organization (WHO) [4]. However, for a durable routine use, GC-IDHRMS requires the extracts subjected to measurement to be of an extremely good quality (e.g. to be free of matrix interferences responsible for MS ion suppression). It is therefore mandatory to implement a robust sample preparation procedure prior the measurement step. However, it is as important to minimize the time and resource consuming aspect of such a complex procedure to allow the validation and use of the method on a routine basis.

Although liquid–liquid extraction (LLE) was early used (and still sometime is) to isolate dioxin from blood (whole, plasma or serum) [5–8], solid-phase extraction (SPE) based on different formats and sorbents has been considered by many groups to complete the extraction step [9–14]. Among the available sorbents, octadecyl bounded silica and polymer-based sorbents are the more widely used due to their large spectra of application and their robustness over a wide range of extraction media. Other extraction techniques based on immunoaffinity chromatography [15], lipophilic gels [16], pressurized liquid extraction (PLE) [17] and sorbent-assisted LLE [18,19] have also been reported but are currently less widely used than SPE that can more easily be automated [20–22].

Because of the non-selective aspect of the extraction step, a clean-up procedure has to be present to further purify the samples and isolate the analytes of interest from co-extracted matrix components. Based on the use of reported efficient sorbents, Lapeza et al. reported in 1986 on an automated apparatus capable of sequentially clean-up series of extracts [23]. The system matured over the years to offer parallel operation mode and finally became one of the most used automated clean-up system in the field of dioxin analysis [24,25]. It is based on the use of disposable columns made of sorbents such as silica, alumina, Florisil, C18, Bio-Beads and dispersed carbon. This apparatus acts as an interferences and fat remover, and is also responsible for the fractionation of the analytes into sub-classes suited for GC separation and MS measurement [26,27]. Its use allows the fast and reliable production of clean extracts for GC–IDHRMS analysis.

The purpose of the present project was to optimize an updated multi-step procedure based on C18 SPE, automated multi-column clean-up and GC–IDHRMS for the measurement of selected PCDD/Fs and PCBs in human serum under strong QA/QC criteria. This was required by our routine laboratory, which is confronted to the following challenges: (1) measurements are to be performed in samples that contain increasing levels of toxicants, (2) available sample sizes are decreasing and (3) the demand in terms of sample throughput is increasing.

2. Experimental

2.1. Chemicals and consumables

Hexane, toluene, methanol and dichloromethane were Picograde reagents (LGC Promochem, Wesel, Germany). Nonane puriss analytical-reagent grade standard for GC was purchased from Fluka (Steinheim, Germany). Formic acid 97–100% puriss analytical-reagent grade was from Riedel-de Haën (Seelze, Germany). Water was obtained from a Milli-Q Ultrapure water purification systems (Millipore, Brussels, Belgium). All solvent batches were tested for contamination before use. Sodium sulfate disposable drying cartridges (Isolute 2.5 g) and octadecyl disposable cartridges (Isolute C18, 2 g/15 ml) had polyethylene bars and frits and were obtained from Argonaut-Sopachem (Brussels, Belgium). Reusable glass columns used for manual PCB clean-up were home-made (20 cm length, 2 cm diameter). Disposable PTFE columns for the automated clean-up were obtained from Fluid Management Systems (FMS, Waltham, MA, USA). Chromatographic pure grade helium gas, 99,9999% was purchased from Air Products (Vilvoorde, Belgium).

Internal standard (I.S.) solutions of the seventeen 2,3,7,8-chloro-substituted and four NO-PCBs 13C12 congeners labelled PCDD/Fs (EDF-4144), the nine-points calibration standard solution (EDF-4143) and the syringe standard (EDF-4145) were purchased from Cambridge Isotope Labs. (Andover, MS, USA). The EDF-4143, EDF-4144 and EDF-4145 concentrations of the natives and labelled congeners are summarised in a previous report [26]. The 13C12-labelled MO-PCB I.S. spiking solution (MBP-MXK) (Wellington Labs., Guelph, Canada) containing the IUPAC PCB 105, 114, 118, 123, 156, 157, 167, 170, 180, 189 was diluted to a concentration of 200 pg/µl. The 13C12-labelled indicator (I) PCB I.S. spiking solution (EC-4058) (CIL) containing the IUPAC PCB 28, 52, 101, 138, 153, 180, 209 was diluted to a concentration of 200 pg/µl. An eight-points calibration standard solution was prepared from individual congeners for MO- and I-PCBs (LGC Promochem). Native concentrations were 0.8, 2, 8, 20, 40, 80, 160 and 280 pg/µl and the 13C were 200 pg/µl. The PCB syringe standard was 13C12PCB 80 at 200 pg/µl. An I.S. solution made of a mixture of the PCDD/Fs and PCBs was prepared to facilitate the spiking procedure of the samples. All standards were in nonane.

Quality control (QC) samples consisted in foetal calf bovine serum fortified with PCDD/Fs and PCBs.

The mean level was 280 pg TEQ/l (157 ± 15 pg TEQ/l for PCDD/Fs, 106 ± 14 pg TEQ/l for NO-PCBs and 16 ± 2.5 pg TEQ/l for MO-PCBs). Internal quality control (IQC) charts and related data treatment were carried out with a dedicated software (MultiQC, quality control software, Metz, France).

2.2. Sample preparation

2.2.1. Sample pre-treatment

QC and unknown serum samples were stored at −20 °C prior to analysis. QC samples were stored in dark bottles containing approximately 20 g of serum although unknown samples were conditioned in drawing bags. The drawing bags had been blank-tested prior to sampling to ensure that they were free of any PCDD/F and PCB contaminations. Samples to be processed were removed from the freezer at the end of the day preceding analysis and left in a dark cabinet for thawing at room temperature overnight. For QCs, the entire (20 g) sample was used for
analysis. For unknown samples, an aliquot of 20 g was taken and the remaining sample was bottled and put back at −20°C as a backup aliquot.

2.2.2. Lipid content determination

Enzymatic lipid determinations of unknown samples were performed by a sub-contractor clinical laboratory on a dedicated 5 ml serum sub-sample. Four types of lipids were targeted and measured: triglycerides, total cholesterol, non-esterified (free) cholesterol and phospholipids B. Sample sizes were as follow: triglycerides (2 μl), total cholesterol (2 μl), non-esterified (free) cholesterol (50 μl) and phospholipids B (20 μl). A well-documented summation method was used to estimate the total lipid concentrations [28]. The total lipid content was expressed in g/l. For the inter-conversion of volumetric and gravimetric data, a value of 1.026 g/ml for serum specific gravity. Lipid concentration of unknown samples processed last 2 years ranged from 4 to 17 g/l.

2.2.3. Manual solid-phase extraction

SPE was carried out using 2 g/15 ml non-endcapped [−Si(Me3)] octadecyl SPE disposable cartridges. Series of 20 samples were split in 2 sub-series of 10 samples each and processed semi-sequentially on 2 separate VacMaster-10 Sample Processing Manifolds (Argonaut-Sopachem) by a single operator. Twenty gram samples were weighed in 100 ml glass bottles and an equal volume of formic acid was added under a fume hood. Samples were gently agitated for few seconds further left to degaze 20 min and an equal volume of water was added was added (60 ml total volume). BC samples consisted in 40 ml of Milli-Q water and 20 ml of formic acid. In the mean time, SPE cartridges were gravimetrically conditioned using two aliquots of methanol (2 × 6 ml) and two aliquots of Milli-Q water (2 × 6 ml). The 60 ml samples were quantitatively added to the cartridges after the I.S. mixture was added on the SPE frits. Two aliquots of 10 ml of Milli-Q water were used to wash the sample bottles and cartridge barrels. Cartridges were dried under water pump suction for 2 h. After removal of all aqueous solvents from the manifold reservoir, dedicated collection glass tubes (20 ml) were placed in the manifolds to collect eluates. The disposable sodium sulfate cartridges were then placed at the bottom of the C18 cartridges. The elution took place using three aliquots of hexane (3 × 5 ml). Those extracts were processed the same day for clean-up and fractionation (see Fig. 1 for complete procedural scheme).

2.2.4. Automated clean-up and fractionation

The clean-up and fractionation in sub-groups of analytes was performed by automated multi-sorbent (silica, alumina and carbon-based) liquid chromatography (LC) using the PowerPrep System (FMS) [24–27]. Sizes of PTFE disposable columns were 4 g (2 g acid, 1 g basic and 1 g neutral) for silica, 4 g for basic alumina and 0.35 g of carbon dispersed on Celite for the carbon column. The total run time was 45 min, plus a preventive decontamination program of 15 min. The MO- and I-PCBs were isolated in 120 ml of hexane–dichloromethane (1:1) (F3) and the PCDD/Fs and NO-PCBs were collected in 80 ml of toluene (F4) (Fig. 2). The volumes of those fractions were reduced to 200 μl in a sensor-equipped TurboVap II Workstation (Caliper Life Science, Teraflene, Belgium) under nitrogen stream. Evaporation temperatures were 20 and 40°C for F3 and F4, respectively. Ten drops of toluene were added to F3 prior reaching the final volume. The 200 μl extracts were transferred in 1.2 ml GC conical vials containing 40 and 5 μl of nonane used as keeper for the hexane–dichloromethane fraction (F3) and toluene fraction (F4), respectively. The GC vials were further evaporated using a RapidVap (Labconco, Kansas City, MO, USA) to remove the remains of toluene and hexane–dichloromethane at 30°C under vortex motion. The pressure was set at 310 and 76 mbar for F3 and F4, respectively. The final volumes were 40 μl for the MO- and I-PCBs fraction (F3) and 5 μl for the PCDD/Fs
and NO-PCBs fraction (F4). Ten microliters and 5 μL of syringe (recovery) standards were added to the GC vials prior to injection for F3 and F4, respectively.

2.2.5. Alternative manual clean-up for PCBs

The alternative procedure for the measurement of I-PCBs is carried out on 10 g sample sizes extracted as described in Section 2.2.3 using SPE. Acid silica was freshly prepared by adding 80 mL of sulfuric acid to 120 g of hexane washed silica gel and by mixing the slurry until homogeneity. Partially deactivated alumina was prepared by adding 10 mL of water to 90 g of alumina oxide and by mixing the slurry until homogeneity. A glass column was hexane wet packed in the following order: a glass wool plug, 4 g acidic silica, 1 g partially deactivated alumina and 0.5 g sodium sulfate. The extract was eluted by gravimetry and the column was rinsed twice with 5 mL of fresh hexane. The evaporation procedure was similar as described earlier.

2.3. Instrumentation

2.3.1. GC–IDHRMS of MO-PCBs and I-PCBs

Measurements of MO-PCBs and I-PCBs (F3) were carried out on a MAT95 XL (ThermoFinniganMAT, Bremen, Germany) connected by a heated transfer line (275 °C) to an Agilent 6890 Series (Palo Alto, CA, USA) gas chromatograph equipped with a A200S autosampler (Thermo). The column was an HT-8 (25 m × 0.22 mm I.D., 0.25 μm df) (SGE, Villebon, France). Helium was used as the carrier gas at constant flow rate of 0.8 mL/min. 1.2 μL of the final extract in nonane (95%) were injected into a split/splitless injector held at 275 °C in splitless mode. Drilled Uniliner (Restek, Evry, France) were used. The oven temperature was maintained at 140 °C for 2 min, ramped at 15.0 °C/min to 220 °C held for 7.5 min, ramped at 6.0 °C/min to 250 °C, ramped at 2.0 °C/min to 265 °C and finally ramped at 28 °C/min to 320 °C. The total separation time was 30 min. The HRMS instrument was operated in the selected ion monitoring (SIM) mode. Two ions were monitored for both native and labels for isotope ratio check. A complete calibration was carried out every month (200 unknown samples) and instrumental blanks were measured for every series of samples. Calibration stability was ensured by injecting a randomly selected single point of the curve with every series of samples. A complete calibration was done in case of a duplicate check point was outside a 95% confidence interval. Additional GC and HRMS parameters were described previously [26].

2.3.2. GC–IDHRMS of PCDD/Fs and NO-PCBs

Measurements of PCDDs, PCDFs and NO-PCBs (F4) were carried out on an Autospec Ultima (Micromass, Manchester, UK) connected by a heated transfer line (275 °C) to an Agilent 6890 Series (Palo Alto) gas chromatograph equipped with a A200SE autosampler. The column was a VF-5MS (50 m × 0.2 mm I.D., 0.33 μm df) (Varian, Sint-Katelijnewave, Belgium). Helium was used as the carrier gas at constant flow rate of 1.0 mL/min. Five microliter of the final extract in nonane (10 μL) were injected into a programmable temperature vaporization (PTV) injector equipped with a PTV multi-baffled liner (Agilent Technologies, Diegem, Belgium). The injector temperature program started at 40 °C for 3 min, the temperature was then ramped at 720 °C/min to 320 °C for 4 min and then ramped at 720 °C/min to 330 °C for 43 min. The oven temperature was maintained at 60 °C for 5 min, ramped at 70.0 °C/min to 200 °C, ramped at 3.2 °C/min to 235 °C for 1.5 min, ramped at 3.2 °C/min to 270 °C for 10 min and finally ramped at 15 °C/min to 310 °C for 13 min. The total separation time was 55 min. The HRMS instrument was operated in the SIM mode. Two ions were monitored for both native and labels for isotope ratio check. A complete calibration was carried out twice a month (100 unknown samples) and instrumental blanks were measured for every series of samples. Additional GC and HRMS parameters were described previously [26].

3. Results and discussion

3.1. Sample preparation

3.1.1. Extraction

The extraction procedure is based on a SPE methodology used at the Centers for Disease Control and Prevention in Atlanta [29]. Following the current levels in the EC general population, we decreased the serum sample size to 20 g to ensure proper detectability of the target analytes using a minimum amount of matrix. Practically, with a 0.5% lipid serum, this represents 0.1 g of lipids. For 2,3,7,8-TCDD, which is present in samples at a level close to 1 pg/g lipid, it corresponds to 100 fg/20 mL. With an 80% recovery rate, and PTV injection of one-half the final extract in GC–IDHRMS (typically 5 μL out of 10 μL), 40 fg are available for measurement, which is close but still above (S/N > 3) our instrumental limits of detection (iLODs). Those iLODs were checked every time a series of 20 samples was analyzed. A standard solution containing 2,3,7,8-TCDD at 20 fg/μL was injected (5 μL injection and 80 fg on column) and the resulting signal-to-noise (S/N) ratio value was compared to a minimum requirement value of 10 (Fig. 3). One can see that, over a period of more than 6 months, the instrumental response was in agreement with our recommendations (average value of S/N = 19).

Although 5 and 10 g C18 cartridges were previously reported for 2–10 and 10–100 g sample weights, we scaled down the sorbent bed size to 2 g to cut down prices and reduce solvent consumption. Non-endcapped [—Si(Me3)] C18 sorbent was used because of its potentiality to create secondary polar interaction and improve the retention efficiency for the highly chlorinated congeners. A comparison between 10 and 2 g C18 bed sizes for 20 g samples also showed that 10–20% larger recovery rates were obtained with the smaller cartridge size together with a better reproducibility. These facts are to be attributed to a larger dispersion of the analytes on the 10 g beds. The volumes of solvents and reagents were reduced to 20 mL of formic acid, 12 mL of methanol and 40 mL of water per sample.

The drying of the sorbent bed was carried out by water pump suction of the C18 cartridges for 2 h to ensure efficient water removal without using centrifugation, which would require additional expensive equipment. Shortening the length of this drying step conducted to incomplete drying. The presence of trace of
water is incompatible with the clean-up procedure that employs deactivated alumina to retain the analytes. The use of a small amount of water miscible organic solvent to improve the water removal was avoided because of the important risk of partially eluting analytes of interests. Disposable sodium sulfate cartridges were used during hexane elution to palliate possible uneven drying between the various positions of the manifold. The sample pre-treatment and extraction step required close to 4 h per series of 20 samples per analyst.

The addition of the multi-analyte $^{13}$C-labelled I.S. (30 μL of nonane) was originally carried out before the addition of formic acid to the sample. The low miscibility of nonane with the aqueous sample however resulted in poor homogeneity (even after proper agitation) and favoured the rejection of the spike onto the sample container wall. This could potentially results in an overestimation of the concentration when performing isotope dilution quantification. One solution was to use a water miscible solvent such as methanol to handle the multi-analyte standard but we tried to stay away from that to avoid evaporation problems during standard use and medium-term storage. We decided to perform the I.S. addition directly on the polyethylene frit right after the conditioning step and before the sample load. Proper transfer of the prepared sample on the frit was ensured by multiple rinse of the sample container. Such an approach is not perfect because the I.S. does not follows the sample from the very beginning but it ensures quantitative transfer of the I.S. to the sample. This procedure permitted to improve recovery rates from 40–50 to 60–70% for blanks. Such recovery rates are similar to the ones of unknown samples and QCs. We recently organized a dedicated inter-laboratory exercise to adequately document the efficiency of the procedure. It clearly appeared that our spiking procedure does not influence the quantification process and that the trueness and accuracy of the measurement are preserved [30].

3.1.2. Clean-up and fractionation

The automated clean-up and fractionation procedure was originally developed to accommodate up to 1 g of lipids for multi-analyte measurement [31]. Some of our early work consisted in its modification for measurement of PCDD/Fs in high fat content biological samples, which require at least 4 g of fat to be processed [26]. For the present application on serum where 20 ml specimen (<0.3 g of lipids) were considered for measurement, the goal was to scale down the column sizes and solvent volumes to reduce blank (BC) levels. Such a reduction of the BCs is targeted to improve limits of quantification (LOQs) of the method and thus minimize the number of non-detectable (nd) and non-reportable (nr) values to facilitate data handling [32,33]. As an example, the most significant changes were observed for PCB 77 and PCB 81 for which blank levels were nearly cut by one-half.

The new silica and alumina column sizes (4 g) allowed a significant reduction of the solvent consumption while maintaining a high level of extract purity. A similar approach was recently carried out to reduce blank issues and decrease analytical costs for the measurement of polybrominated diphenyl ethers (PBDEs) in fish samples [34]. As mentioned earlier, the lipid content of unknown serum samples processed during the last 2 year studies of our laboratory varied between 4 and 17 g/l. It corresponds to minimum and maximum values of 0.08 and 0.34 g of lipids that entered the clean-up system. An example of chromatographic quality is illustrated in Fig. 4 for a sample with low level of 2,3,7,8-TCDD and a relatively high content of lipids (9 g/l). Peaks of targeted ions can be identified and quantified...
Fig. 4. Unknown human serum sample chromatogram (selected ion monitoring) showing the characteristic mass windows for 2,3,7,8-TCDD isotopic dilution analysis ($t_R = 29.7$ min). The top two traces represent the $^{12}$C natives and the bottom two traces represent the $^{13}$C labels. The native traces represent 19 fg of 2,3,7,8-TCDD injected (0.3 pg/g lipid in sample). Peaks were non-smoothed. S/N was calculated based on noise present prior to the peak of interest and in an time interval equal to five times the peak width at one-half height. The calculation was based on peak-to-peak averaging with rejection of peaks of intensity outside a 2SD interval.

despite the presence of noticeable chemical noise and matrix effects.

Furthermore, the system conditioning and washing steps were compressed. The total solvent consumption for the clean-up and fractionation of one sample extract was 260, 120 and 130 ml for hexane, dichloromethane and toluene, respectively. This represents a reduction of 20% for dichloromethane and toluene and a reduction of 40% for hexane, compared to previously reported methods [31] or a solvent reduction of 230 l per 1000 samples. The processing time for 10 samples in parallel was 60 min, including a 15 min preventive decontamination program, speeding-up the processing time of 20%. In those conditions, the clean-up and fractionation consisted in the quickest part of the entire analytical procedure (Fig. 1). The MO- and I-PCB fraction (F3, 120 ml of hexane–dichloromethane, 1:1) and the PCDD/F and NO-PCB fraction (F4, 80 ml of toluene) were concentrated using automated evaporators. Temperature and flows were set to minimize the loss of the more volatile analytes during evaporation. For the concentration of F3, the procedure included the addition of 250 µl of toluene once the volume of hexane–dichloromethane (1:1) attained 500 µl to ensure proper analyte detainment prior to the addition of nonane as the keeper. The total evaporation time was 2.5 h.

3.2. Comprehensive versus separated approach

3.2.1. Estimation of the limits of quantification

There is currently still a lot of debates among analysts regarding LOD and LOQ definitions for ultra-trace measurement of dioxins. According to the EU dioxin Directive 2004/44/EC [35], the LOQ value is defined as the concentration of an analyte in the extract of a sample which produces an instrumental response at two different ions, to be monitored with a S/N ratio of 3:1 for the less sensitive signal and fulfillment of the basic requirements such as, e.g. retention time, isotope ratio according to the determination procedure as described in EPA [US Environmental Protection Agency] method 1613 revision B. Despite the fact that end-users know that, for real sample extracts (with ‘real noise’), it is somewhat difficult to simultaneously fulfill both the retention time and the isotope ratio requirements with a S/N of 3:1, this definition provides very low LOQ values. The weakness of this definition is that there are no requirements regarding the analytical performances to be
reached (e.g. trueness and precision). Therefore, it is clear that it is tempting for laboratory managers to use those low LOQ values as a commercial argument to point out the sensitivity of a method.

From ‘the fitness for purpose of analytical methods’ IUPAC recommendation [36], LOQ is defined as the ability of a chemical measurement process to adequately quantify an analyte, with ‘the ability to quantify’ expressed in terms of the signal or analyte that will produce estimates having a specified relative standard deviation (RSD), commonly 10% (with an acceptable level of trueness). It is clear that the calculation of LOQs on the basis of the dioxin Directive 2004/44/EC [35] only conducts to analytical performances that do not fit with the requirement listed by the IUPAC recommendation. As an example, replicate measurements ($n = 6$) of 2,3,7,8-TCDF in a QC serum for which the level of the analyte is just above the LOQ (2.1 pg/l of serum, calculated using Directive 2004/44/EC [35] guidelines) had a standard deviation (SD) equal to 0.5 pg/l, with a corresponding RSD of 23%. This is much larger than the recommended 10% RSD and the situation can be as bad as 50–100% RSD for selected congeners at the LOQ level. Additionally, one has to note that it is very difficult to assess the trueness of the method at such ultra-trace levels.

### 3.2.2. The central role of the procedural blank in the determination of LOQs

Additionally to the analytical performance problem, it is recognized that the presence of background contamination severely penalizes the LOQ calculation. In most laboratories, procedural blanks are systematically contaminated with some PCDD/F congeners and, in an even larger extend, with some PCBs. The global world PCB contamination has resulted in the large distribution of these persistent compounds in the environment and their ubiquitous presence in ambient air, solvents and reagents. Even when series of measures are taken to lower their presence in the laboratory environment, the remaining background contamination of several congeners makes analytical chemist life difficult, especially when analyzing low volumes of serum samples at the sub-ppt level. If this background contamination cannot be totally eliminated, the calculation of the LOQs must take it into account. Additionally, it is also recommended to systematically apply the subtraction of the blank concentration to the concentration value measured for unknown samples.

During the course of routine serum analysis, we analyzed lots procedural blanks that were enclosed within series of unknown samples. The relative distribution of PCDD/F and DL-PCB congeners in those procedural blanks remained relatively constant during a period of 18 months. Table 1 lists levels found in procedural blanks on a congener basis. The variability study indicated that the background level of the blanks was under control for the entire study time. RSDs below 60% were achieved, excepted for PCB 81 for which a fluctuating concentrations were recorded. The average values were calculated after rejection of outliers that were outside a +2SD confidence interval, as we assumed a normal distribution of the blank values. Even if we assume that we control the blank level in the case of large RSDs, the high background contaminations observed for PCB 77, 105 and 118 pose a serious problem in terms of LOQ calculations. For those congeners affected by blank contamination, the Directive 2004/44/EC definition [35] is no longer applicable. The LOQ calculation approach has to rest on the estimation of the minimum amount of each congener that can reliably be distinguished from the average amount present in the procedural blank, even if ‘LOQs’ based on instrumental performances only are much lower.

For example, in the case of HxCDFs, only the 1,2,3,4,7,8-HxCDF congener is constantly present in blanks (8.2 pg/l, SD = 0.85 pg/l, RSD = 10.4%). Therefore, although the LOQs for the other HxCDFs (1,2,3,6,7,8-, 1,2,3,7,8,9- and 2,3,4,6,7,8-) are calculated to be 5 pg/l serum (Table 2) based on Directive 2004/44/EC guidelines [35], LOQ calculations for 1,2,3,4,7,8-HxCDF have to include the influence of the background contamination of the analytical procedure. In concrete terms, a one-tailed normal distribution of the blank levels can be assumed (Fig. 5). Hence, a LOQ value of $8.2 + (1.64 \times 0.85) = 9.6$ pg/l or $8.2 + (2.33 \times 0.85) = 10.2$ pg/l is proposed at 95 and 99% level of confidence, respectively. The choice between those two values depends on the acceptable level of risk the analyst is ready to take but, as dioxins are contaminants (by contrast with drug residues) and classified as class B substances in the EU Directive 1996/23/EC [37], a 95% level of confidence is recommended. For this congener, the presence of a background contamination doubles the value of the LOQ initially defined on S/N approach. The difference between LOQ values calculated both ways can be as large as one or two orders of magnitude for some congeners, depending on the blank level and its variability.

### 3.2.3. Manual clean-up option for selected PCBs

As mentioned earlier in the text, high LOQ values are observed for some selected PCBs (Table 2). This is due to elevated BC levels because of the, laboratory environment, sol-

<table>
<thead>
<tr>
<th>Congeners</th>
<th>Average (pg/µl)</th>
<th>SD (pg/µl)</th>
<th>RSD</th>
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<tr>
<td>1,2,3,4,6,7,8-HeptaCDD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.032</td>
<td>0.009</td>
<td>27</td>
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<tr>
<td>OCDD&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.057</td>
<td>42</td>
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<tr>
<td>1,2,3,4,7,8-HexaCDF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.016</td>
<td>0.002</td>
<td>10</td>
</tr>
<tr>
<td>1,2,3,4,6,7,8-HeptaCDF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.032</td>
<td>0.007</td>
<td>23</td>
</tr>
<tr>
<td>OCDF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.079</td>
<td>0.014</td>
<td>18</td>
</tr>
<tr>
<td>PCB 77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.640</td>
<td>0.940</td>
<td>26</td>
</tr>
<tr>
<td>PCB 81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.553</td>
<td>0.669</td>
<td>121</td>
</tr>
<tr>
<td>PCB 126&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.135</td>
<td>0.049</td>
<td>36</td>
</tr>
<tr>
<td>PCB 169&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>0.003</td>
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<tr>
<td>PCB 105&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.139</td>
<td>0.670</td>
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<tr>
<td>PCB 114&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>44</td>
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<tr>
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<td>2.355</td>
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<tr>
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<tr>
<td>PCB 157&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.008</td>
<td>45</td>
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<tr>
<td>PCB 167&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.063</td>
<td>0.025</td>
<td>39</td>
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<sup>a</sup> Final volume of 10 µl.  
<sup>b</sup> Final volume of 50 µl.
vents and consumables. It makes ultra-trace level measurement difficult for some analytes and produces a significant number of undesirable non-reportable congeners. Fortunately, this only has an impact for few congeners, e.g. 77, 105, 118, 28, 52 and 101. Levels of the NO-PCB 77 are extremely low in concentration inside the samples and their TEF are also low, which means that their absence in the TEQ calculation is not critical. The elevated LOQs for MO-PCB 105 and 118 have no real impact because those congeners are present at levels above those LOQs for the general European population. The non-TEF PCB congeners (the I-PCBs 28, 52, 101, not shown in Table 2) are not involved in dioxin-like toxicity calculations. Nevertheless, if levels have to be reported for those PCBs, we have a quick manual PCB method (see Section 2) that can be carried out in parallel to the comprehensive method on 5 ml of serum. The smaller sample size permits to reduce solvent volumes and size of consumables, thus reducing blank levels and LOQs. In practice, after analyzing several hundreds of samples, it appears that spending time and resources cleaning-up extra sample aliquots to get values for those congeners (PCB 28, 52, 101) might not be necessary in all studies because the problematic congeners represent only few % (<10%) of the total PCB concentration.

### 3.3. Multi-levels quality control

The basic approach to IQC involves the analysis of control materials alongside the routine test samples. The main objective is to ensure the constancy of the results on a daily basis, as well as their conformity with strictly defined criteria. An analytical result is characterized by a systematic error and a random error. Although a conventional Shewhart chart does well in terms of random error monitoring, it has a certain lack of sensitivity to detect systematic errors. The exponentially weighted moving average (EWMA) control chart is better suited for this purpose. It is a specific method for improved bias detection and it is defined as ‘a statistic for monitoring the process that averages the data in a way that gives less and less weight to data as they are further removed in time from the current measurement’ [38]. A parameter λ, called the smoothing factor, determines the rate at which ‘older’ data enters into the calculation of the EWMA statistic. Fig. 6 illustrates the IQC charts. In Fig. 6A–C, the central tangent line defines the mean value ($m$) with the upper and lower control limits drawn in plain. The control limits are set at $m \pm 3\sigma_M$, where $\sigma_M$ is the standard deviation recalculated each time a new data point is added in the dataset (floating chart). The tick curve with its control limits (dashed lines, $m \pm 3\sigma_{EMWA}$) represents the EWMA with a smoothing factor of 0.2. The relationship between $\sigma_M$ and $\sigma_{EMWA}$ is expressed by the following equa-
Fig. 6. Internal quality control (IQC) charts for PCDD/Fs (A), NO-PCBs (B) and MO-PCBs (C) present in QC serum analyzed over time (concentrations in pg WHO-TEQ/l). (D) The Hotelling index (see text for details).

In addition, the EWMA curves lay between the dashes lines demonstrating that the bias did not exceed 5.9% (see Eq. (2)) for the sum of PCDD/Fs; 6.7% for the sum of NO-PCBs and 9.8% for the sum of MO-PCBs.

Although the multi-levels IQC is frequently used in clinical chemistry, its application in ultra-trace analysis is rare. The classical approach of multi-levels IQC is characterized by one parameter controlled at three different levels: high, medium and low. The correlation between the levels has been introduced and developed by Hotelling [39]. He defined an index that combines dispersion information, means and correlation of several variables. This scalar, known as $T^2$, generalizes at $p$ dimensions the Student’s $t$-test. This concept and the underlying statistics were used and adapted in the field of dioxin analysis. Instead of measuring each congener at different IQC levels, we divided the 29 toxics congeners into 3 sub-groups and we monitored 3 parameters: the sum of PCDD/Fs, the sum of NO-PCBs and the sum of MO-PCBs, all expressed in TEQ units. The IQC levels were, respectively, 156.2 pg TEQ/l, 106.7 pg TEQ/l and 16.1 pg TEQ/l for PCDD/Fs, NO-PCBs and MO-PCBs, as indicated in Fig. 6. I-PCBs were not screened in this example. The selection of the three sub-groups was related to our analytical procedure for clean-up, even if PCDD/Fs and NO-PCBs were collected in the same fraction. Once injected in parallel into the two GC–IDHRMS, the three monitored parameters were quantified and used to build up multi-level IQC charts (Fig. 6). As the three variables came from the same analytical procedure, a degree of correlation between control levels should be observed. In other words, when a level (e.g. PCDD/Fs) increases, the cor-

$$\sigma_{EMWA} = \sigma_M \sqrt{\frac{\lambda}{2 - \lambda}}$$

(1)

By setting $\lambda = 0.2$, Eq. (1) becomes:

$$\sigma_{EMWA} = \frac{\sigma_M}{3}$$

(2)

Hence, the dashed lines represent $m \pm \sigma_M$.

The IQC used for this study was a matrix quality control material (foetal calf bovine serum) that was characterized by a sufficient homogeneity and stability for long-term control of laboratory performances. A batch of three litres of serum was prepared to cover 1 year period. The batch was spiked with dioxins, furans and dioxin-like PCBs (see Section 2.1). More than 90 tests were performed during this period including homo-

Fig. 6. Internal quality control (IQC) charts for PCDD/Fs (A), NO-PCBs (B) and MO-PCBs (C) present in QC serum analyzed over time (concentrations in pg WHO-TEQ/l). (D) The Hotelling index (see text for details).
responding levels (e.g. NO-PCBs and MO-PCBs) should follow the same trend. This was quantified by Hostelling’s $T^2$ test. A $T^2 > 1$ gives a warning value. It indicates that an anomaly is detected even if each individual control material is in-control. As an example, Fig. 6D shows a detected even if each individual control material is in-control. the same trend. This was quantified by Hostelling’s responding levels (e.g. NO-PCBs and MO-PCBs) should follow $10^6$ J.-F. Focant et al. / J. Chromatogr. A 1130 (2006) 97–107

4. Conclusions

The reported procedure permits the high-throughput semi-automated measurement of PCDDs, PCDFs and PCBs, in human serum samples. The entire procedure is accredited according to ISO 17025 [40] and in agreement with EU QA/QC recommendations [41] regarding blank controls, sensitivity, LODs, LOQs, trueness, precision and inter-laboratory studies for dioxin analysis. IQC EWMA charts based on ‘in-house’ fortified bovine serum QC pool show random distribution of data points over time and operators. In terms of turnover, once series keep being taken by means of checking blank level, recovery calculations, integration of the peaks, peak shape, retention time, isotope ratio and relative response factor to ensure that was an isolate case of deviation.

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References

