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Abstract An accurate and easy method for the extraction, cleanup, and HRGC-HRMS analysis of dioxin-like PCBs (DL-PCBs) in low-volume serum samples (1 mL) was developed. Serum samples were extracted several times using *n*-hexane and purified by acid washing. Recovery rates of labeled congeners ranged from 70 to 110 % and the limits of detection were below 1 pg/g on lipid basis. Although human studies are limited and contradictory, several studies have shown that DL-PCBs can have adverse effects on the male reproductive system. In this way, the present method was applied to 21 serum samples of male patients attending fertility clinics. The total levels obtained for the patients ranged from 6.90 to 84.1 pg WHO-TEQ/g lipid, with a mean value of 20.3 pg WHO-TEQ/g lipid. The predominant PCBs (the sum of PCB 118, 156, and 105) contributed 67 % to the mean concentration of total DL-PCBs in the samples analyzed.

Keywords separated by ' - ' Human serum - Male infertility - DL-PCBs - HRGC-HRMS - Pollutants - Analytical method

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RESEARCH ARTICLE

Levels of dioxin-like PCBs in low-volume serum samples of male patients attending fertility clinics

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Abstract An accurate and easy method for the extraction, cleanup, and HRGC-HRMS analysis of dioxin-like PCBs (DL-PCBs) in low-volume serum samples (1 mL) was developed. Serum samples were extracted several times using *n*-hexane and purified by acid washing. Recovery rates of labeled congeners ranged from 70 to 110 % and the limits of detection were below 1 pg/g on lipid basis. Although human studies are limited and contradictory, several studies have shown that DL-PCBs can have adverse effects on the male reproductive system. In this way, the present method was applied to 21 serum samples of male patients attending fertility clinics. The total levels obtained for the patients ranged from 6.90 to 84.1 pg WHO-TEQ/g lipid, with a mean value of 20.3 pg WHO-TEQ/g lipid. The predominant PCBs (the sum of PCB 118, 156, and 105) contributed 67 % to the mean concentration of total DL-PCBs in the samples analyzed.

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Introduction

The possible relationship between environmental and occupational exposure to toxic substances and the decreasing quality of semen and the consequent increase in male infertility is a subject of great scientific interest (Rubes et al. 2005). Several laboratory animal studies have indicated that some endocrine disruptors as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) may affect human reproduction and may be linked to reduced fertility in women and men (Cok et al. 2008).

The World Health Organization has estimated that nearly 8 % of couples around the world present some forms of infertility problems during their reproductive lives. This means that 50 to 80 million people suffer fertility problems (Woodruff et al. 2010). Specially, there are evidence for decreasing quality of semen during the last decades (Carlsen et al. 1992). Environmental factors (exposure to traffic exhaust fumes, dioxins, combustion products, etc.) and several lifestyles as obesity and smoking could negatively affect human fertility, underlining the importance of environmental and lifestyle impacts throughout the life course (Sharpe 2010).

Their lipophilic nature, low biodegradability, and chemical stability, together with the indiscriminate use of dioxin-contaminated PCBs, ensure their ubiquity and persistence in the environment (Esposito et al. 2014). Exposure of the general population to dioxin-like PCBs occurs mainly through food, and also dermal contact and inhalation. In order to quantify trends and patterns of human exposure to chemical agents, monitoring programs play a fundamental role (Angerer et al. 2007; Needham et al. 2007; Patterson et al. 2009; Viso et al. 2009). Moreover, monitoring programs act as a framework to evaluate health impacts and the effectiveness of public and private policies aimed at decreasing exposure to POPs (Department of Health and Human Services 2009; Viso et al. 2009).

There is little information on infertility and related conditions on human exposure to DL-PCBs, most of the papers published are relating to the presence of others PCBs congeners in different fluids of the human reproductive tract such as the seminal fluid, cervical mucus, and follicular fluid.

Nowadays, there is an increasing demand to reduce the size of the specimen collected for human biomonitoring (Patterson et al. 2011). In this way, there are several studies analyzing the DL-PCBs content in serum samples from different group of populations; however, there are scarce studies reporting the analysis of serum from individuals using low-volume samples, due to the difficulty in reaching the required selectivity and sensibility for the analysis of these compounds.

The goal of this work is to develop an analytical method for the analysis of small-volume human serum samples (1 mL) in order to provide an individualized patient study. The optimization of the analytical method has been carried out considering these important aspects: minimizing both the consumption of chemical reagents and analysis time, in order to minimize the cost of the analysis, but keeping the quality standards required for the analysis of dioxin-like PCBs.

Methods and materials

In this study, 21 blood serum samples were collected from male patients recruited for assisted reproduction at the IVF Spain Treatment Clinic from May 2012 to June 2014. The age of the men ranged from 30 to 55 years and the body mass index (BMI) ranged between 21.0 and 31.8 (mean 25.2). General information regarding age, place of residence, smoking habits, dietary habits, and alcohol consumption, along with occupational, residential, and clinical histories were collected. Each patient provided an informed consent after receiving a detailed explanation of the study and potential consequences and signed an informed consent form.

Blood samples were obtained by venipuncture and collected in Vacutainer test tubes without anticoagulant and were immediately transferred to a glass centrifuge tube. After centrifugation and decantation, the serums were stored in the dark at −20 °C until analysis.

The procedure used for analytical determination was adapted from a previously published method (Grimalt et al. 2010) for the analysis by gas chromatography with electron capture detection of several halogenated organic pollutants, including seven PCB congeners (PCBs 28, 52, 101, 118, 138, 153, and 180).

From an analytical point of view, gas chromatography (GC) coupled with magnetic sector high-resolution mass spectrometry (HRMS) is the most sensitive and specific technique in the field of ultra-trace measurement of dioxin-like PCB. The use of isotope dilution methodology, based on ^{13}C-labeled standards at a minimum mass resolution of 10,000 resolving power in the selected ion monitoring (SIM) acquisition mode, allows enhanced selectivity and the lowest sensitivity threshold (Patterson et al. 2011). In this way, in this study, the extraction and cleanup with *n*-hexane and concentrated sulfuric acid was followed by HRGC-HRMS determination. Figure 1 shows the analytical procedure optimized for the determination of DL-PCBs in low-volume samples of human serum. To improve the separation between aqueous and organic phases, 10 drops of Milli-Q water were added before sample centrifugation.

The samples were analyzed using an Agilent HP5890 gas chromatograph equipped with programmable temperature vaporization (PTV) inlet, coupled to a Micromass Autospec Ultima-NT mass spectrometer. An Agilent DB5-MS chromatographic column (60 m×0.25 mm i.d.×0.25 μm) was used for the analysis. Isotopically labeled standards were obtained from Wellington Laboratories Inc. (Canada). *n*-Hexane and sulfuric acid were for organic trace analysis and were purchased from Merck (Germany).

Concentration of lipophilic pollutants in serum are usually expressed on a lipid weight basis instead of on a fresh weight basis, with a view to obtain biomarker values that reflect body burden of persistent organohalogen pollutants. In this work, the total lipid concentration was determined by the sum of the triglyceride and cholesterol concentrations, according to the following regression (Phillips et al. 1989):

Total lipid (mg/dL)=62.3+triglycerides (mg/dL)+2.27∗ total cholesterol (mg/dL)

The levels of dioxin-like PCBs are expressed in picograms of compound per gram of lipid (pg/g lipid) and the toxic equivalents were calculated according to the toxic equivalency factors published in 2005 by the World Health Organization (WHO_{2005}-TEFs) (van den Berg et al. 2006).

Samples were spiked with ^{13}C-labeled internal standards before extraction and with ^{13}C-labeled recovery standards before HRGC-HRMS analysis. Recovery rates of labeled congeners ranged from 70 to 110 % and the limits of detection were below 1 pg/g on lipid basis. Quality assurance criteria were based on the minimum requirements described in US EPA method 1668C for dioxin-like-PCBs (US EPA 2010). A procedural blank was associated with each batch of four samples and processed in the same manner. The congeners below the limit of detection (LOD) were calculated considering a concentration equal to their respective LOD.

Results and discussion

In this study, DL-PCBs have been determined in 21 serum samples from male patients recruited for assisted reproduction. Ten patients aged between 30 and 40 years and the rest aged between 41 and 55 years. The BMI was categorized into three groups: BMI <25, not overweight; BMI 25-29.9,

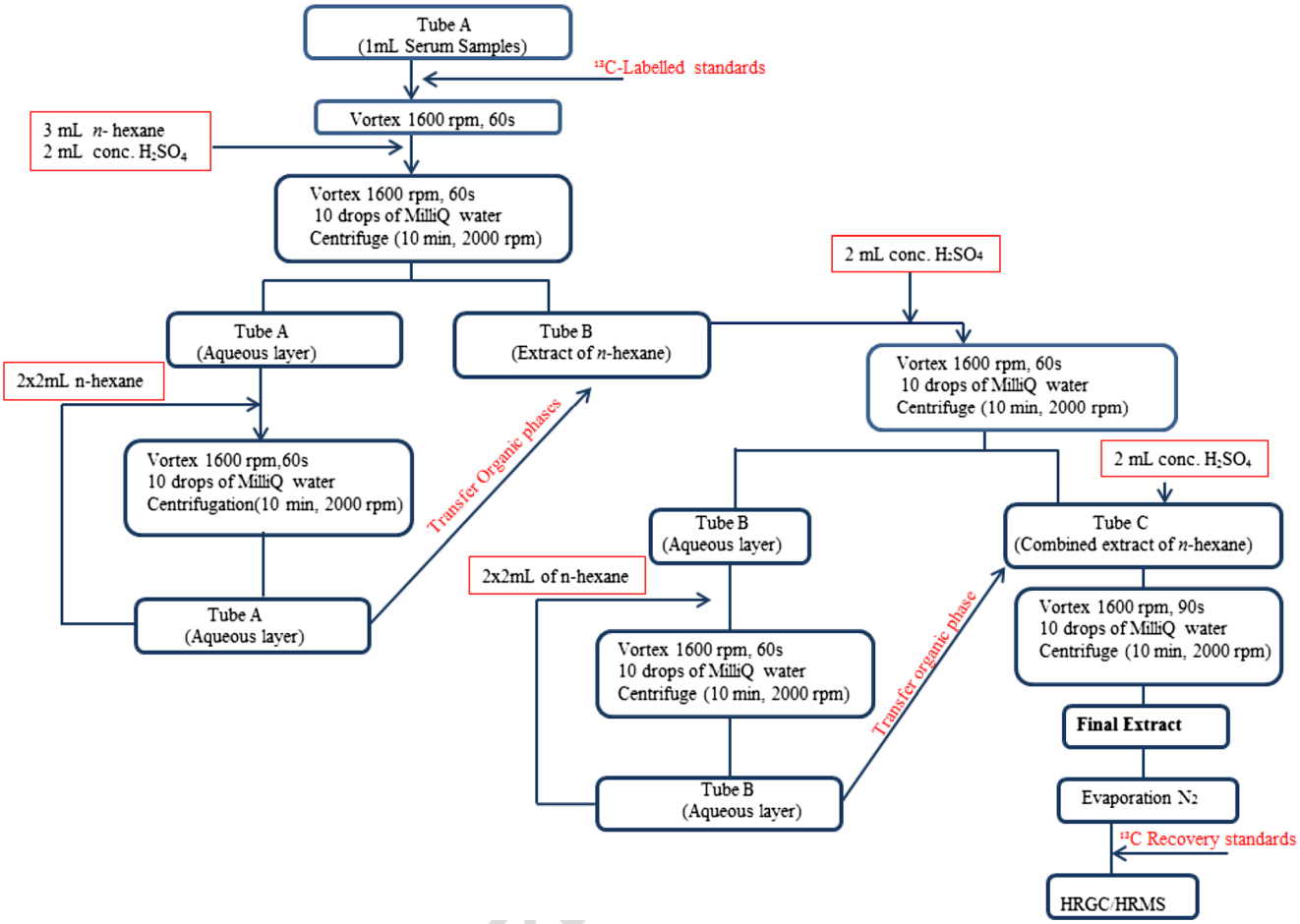

Fig. 1 Scheme of the analytical procedure developed

Table 1 Levels of DL-PCB congeners in the blood serum, expressed as pg/g lipid, and total DL-PCB concentrations, expressed as pg WHO-TEQ/g lipid

	Mean	Median	Standard deviation	Minimum	Maximum	% above LOD	% WHO-TEQ
Non-ortho dioxin-like PCBs							
PCB 77	230	147	254	18.7	1110	81	0.14
PCB 81	72.5	61.5	53.8	14.3	250	19	0.15
PCB 126	138	88.9	156	41.2	638	57	65.6
PCB 169	211	109	232	56.1	1010	81	30.1
Mono-ortho dioxin-like PCBs							
PCB 105	527	394	315	137	1040	100	0.11
PCB 114	147	117	100	34.2	387	76	0.03
PCB 118	2170	1740	1270	462	5220	100	0.47
PCB 123	138	87.8	94.3	23.0	368	62	0.03
PCB 156	1080	886	716	196	2630	100	0.25
PCB 157	269	227	180	85.5	823	95	0.06
PCB 167	322	262	212	66.6	788	95	0.07
PCB 189	345	244	337	103	1690	95	2.95
Sum							
DL-PCB	5650	5210	2780	2020	13,200		
WHO-TEQ DL-PCB	20.3	11.6	22.4	6.90	84.1		

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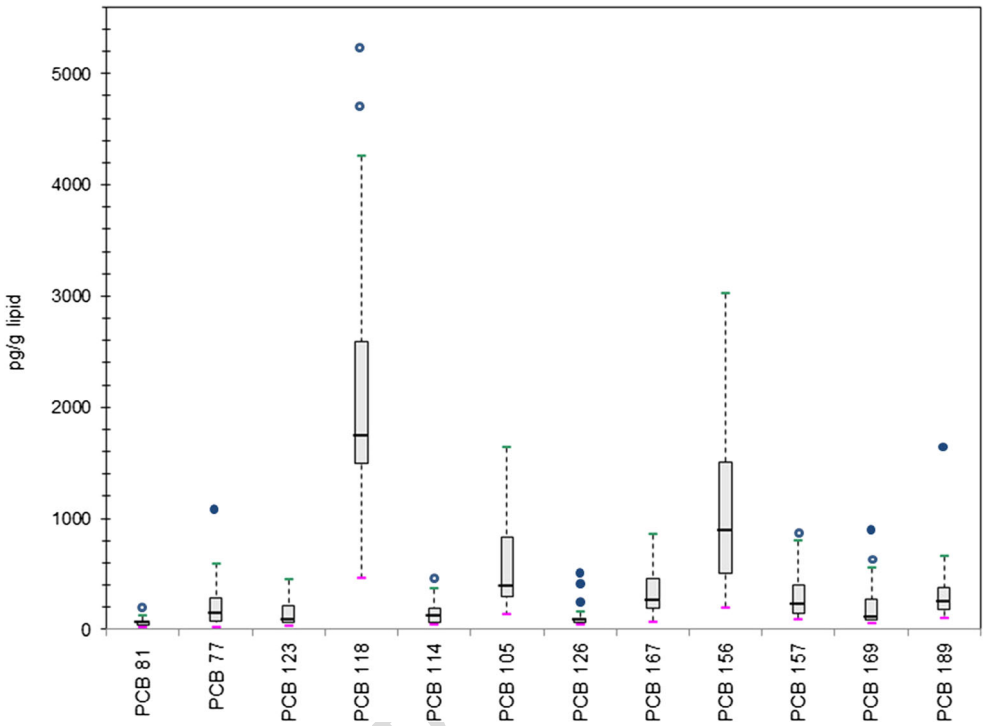

Fig. 2 Boxplots of median values of DL-PCBs

overweight; and BMI ≥30, obese. Only one patient had a BMI >30, while for the rest of the patients, the number of not overweight and overweight was the same. To describe the levels of DL-PCBs, we calculated the medians and arithmetic means, their standard deviations, the maximum and minimum values, and the percentage of samples that present a concentration above the limit of detection. An overview of the values obtained is shown in Table 1.

The total levels of DL-PCBs obtained for the patients ranged from 6.90 to 84.1 pg WHO-TEQ/g lipid, with a mean value of 20.3 pg WHO-TEQ/g lipid. The predominant congeners, in order, were PCB 118, PCB 156, and PCB 105, with mean values of 2170, 1080, and 527 pg/g lipid, respectively. The predominant PCBs (the sum of the three previously mentioned) contributed 67 % to the mean concentration of total DL-PCBs in the samples analyzed. Two congeners, PCB 126 and PCB 169 contributed 96 % to the total WHO-TEQ value, due to their high toxic equivalency factors. The mean value for non-ortho PCBs was 20.2 pg WHO-TEQ/g lipid and for mono-ortho PCBs 0.53 pg WHO-TEQ/g lipid. All the congeners were detected in the samples, although PCB 81 was detected only in 4 of the 21 samples analyzed. PCBs 118, 156, and 105 were detected in all the serum samples.

The total TEQ at the 90th percentile of the US population in NHANES 2003–2004 was 30.0 pg/g of lipid (Patterson et al. 2009) for total dioxin-like compounds (PCDD/Fs and DL-PCBs). Wittsiepe et al. (2007) found a total dioxin-like compounds value of 28.36 pg TEQ/g lipid in the blood from pregnant women. In a review of worldwide literature (works published from 1989 until June 2010) (Consonni et al. 2012) in general populations not directly exposed to recognized emission sources, a mean value of 3.7 pg WHO-TEQ/g lipid for the mono-ortho and non-ortho PCBs is given. Several authors have studied the DL-PCBs in blood serum samples from populations living in industrial areas or near urban waste treatment or incineration plants. Zubero et al. (2009) compared the DL-PCBs in serum samples of different populations and found that the mean values ranged between 4.1 and 15.6 pg WHO-TEQ/g lipid; the values obtained for the male patients facing fertility problems are higher than those presented by these studies.

Boxplots with whiskers for DL-PCBs congeners distribution are shown in Fig. 2. Boxes represent interquartile ranges (IQR), medians are marked in each box with a line, the whisker on the appropriate side is taken to 1.5·IQR from the quartile (the "inner fence") and individual outlying data points are displayed as unfilled circles (for suspected outliers) or filled circles (for outliers). Suspected outliers are above 1.5·IQR and individual outlying data points are above 3·IQR. There are two serum samples analyzed that present several outliers values for different isomers, one serum for PCB 169, PCB

Table 2 Concentratios of DL-PCBs (p WHO-TEQ/g lipid) in male serum samples by age and BMI

	Mean	Median	Minimum	Maximum
30–40 years	19.2	11.5	7.40	84.2
41–55 years	21.3	11.4	6.90	83.9
BMI <25	29.9	17.8	6.90	84.2
BMI 25–29	11.9	11.1	7.40	19.2

189, and PCB 126 and another sample for PCB 81, PCB 77, and PCB 126.

Table 2 summarizes the concentrations of DL-PCBs (pg WHO-TEQ/g lipid) in male serum samples by age and BMI. Generally, the concentration of PCBs in human samples tended to increase with age (Chen et al. 2013), which is consistent with the fact that these compounds are liposoluble substances that accumulate in the adipose tissue. The results obtained in this study are in accordance with this general trend, although the values are quite similar in the two groups of age. The role played by BMI has not yet been established and there is controversy with respect to the direction of its association. One of the main findings is that some of the PCBs are positively related to fat mass, while others show an opposite association. Typically the low-chlorinated PCBs were related to fat mass in a positive way, while the opposite trend was generally seen for the highly chlorinated compounds (Ronn et al. 2011). In this study, overweight subjects had much lower mean values than the normal weight patients; this could be explained by the distribution of a given body burden in a higher amount of fat, so the lipid concentration is lower in the overweight patients.

PCBs have been associated with a range of adverse health effects, including adverse effects on reproduction. However, the human data available on this topic remain vague and largely inconclusive (Meeker et al. 2011). Although there are several studies that try to relate the serum concentrations of PCBs (mainly analyzed by GC-ECD) with male and female fertility problems, we have not found any study about the levels of DL-PCBs in male serum samples of patients facing fertility problems, and that makes the comparison of our results a quite complicated task. Dallinga et al. (2002) analyzed PCB 118, 153, 138, and 180 in blood samples from a group of men with very poor semen quality and another group with normal semen quality in order to determine whether a difference in these levels could be established and found a significant relationship between the combined levels of these PCBs and the corresponding level in seminal plasma.

In this work, an analytical method for the analysis of dioxin-like PCBs in low volume (1 mL) serum samples has been carried out, obtaining an easy and cost-effective method with appropriate detection limits. This method has allowed to obtain the amount of DL-PCBs present in the serum of 21 men attending fertility clinics who suffer different fertility problems. Despite the limitations of our study, to the best of our knowledge, this is the first study to investigate the levels of DL-PCBs in the serum of male patients facing fertility problems. Further number of samples, having a better understanding of the lifestyle of patients, and knowing other factors that could affect the relationship between these pollutants and fertility problems could be very useful. It would also be interesting to compare the results of this study with serum samples from fertile patients.

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Compliance with ethical standards

Consent to participate Each patient provided an informed consent after receiving a detailed explanation of the study and potential consequences and signed an informed consent form.

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