RESEARCH PAPER

Determination of selected endocrine disrupting compounds in human fetal and newborn tissues by GC-MS

Achille Cappiello · Giorgio Famiglini · Pierangela Palma · Veronica Termopoli · Anna Maria Lavezzi · Luigi Matturri

Abstract Endocrine disrupting compounds (EDCs) include organochlorine pesticides (OCPs), organophosphate pesticides (OPPs), carbamate pesticides, and plasticizers, such as bisphenol A (BPA). They persist in the environment because of their degradation resistance and bioaccumulate in the body tissues of humans and other mammals. Many studies are focused on the possible correlation between in utero exposure to EDCs and adverse health hazards in fetuses and newborns. In the last decade, environmental pollution has been considered a possible trigger for Sudden Infant Death Syndrome (SIDS) and Sudden Intrauterine Unexplained Death Syndrome (SIUDS), the most important death-causing syndromes in fetuses and newborns in developed countries. In this work, a rapid and sensitive analytical method was developed to determine the level of OCPs and OPPs, carbamates, and phenols in human fetal and newborn tissues (liver and brain) and to unveil the possible presence of non-targeted compounds. The target analytes where selected on the basis of their documented presence in the Trentino-Alto Adige region, an intensive agricultural area in northern Italy. A liquid-solid extraction procedure was applied on human and animal tissues and the extracts, after a solid phase extraction (SPE) clean-up procedure, were analyzed by gas chromatography coupled to a quadrupole mass spectrometric detector (GC-qMS). A GC-TOFMS (time-of-flight) instrument, because of its higher full-scan sensitivity, was used for a parallel detection of non-targeted compounds. Method validation

included accuracy, precision, detection, and quantification limits (LODs; LOQs), and linearity response using swine liver and lamb brain spiked at different concentrations in the range of 0.4–8000.0 ng/g. The method gave good repeatability and extraction efficiency. Method LOQs ranged from 0.4–4.0 ng/g in the selected matrices. Good linearity was obtained over four orders of magnitude starting from LOQs. Isotopically labeled internal standards were used for quantitative calculations. The method was then successfully applied to the analysis of liver and brain tissues from SIUDS and SIDS victims coming from the above mentioned region.

Keywords Endocrine disrupting compounds (EDCs) · Sudden Intrauterine Unexplained Death Syndrome (SIUDS) · Sudden Infant Death Syndrome (SIDS) · Gas chromatography-mass xpectrometry (GC-MS) · Fast-GC · TOF (time-of-flight) MS

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Introduction

Endocrine disrupting compounds (EDCs) are an extensive class of chemicals that may interfere with the natural biological function of hormones by blocking, mimicking, displacing, or acting to subvert their natural roles in living species, including humans. These chemicals include several groups of pesticides and plasticizers, such as organochlorine pesticides (OCPs), organophosphate pesticides (OPPs), phthalates, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), brominated flame retardants (BFRs), perfluorinated compounds (PFCs), and phenols. Most of them are ubiquitous and persistent in the environment, bioaccumulate in the food chain, and can be stored in the body fat where they are slowly metabolized and excreted [1–6]. Owing to these properties, literature data demonstrate an association between EDCs exposure and human health

hazard. The exposure to these compounds can cause several persistent or transient damages in adults, whereas it can lead to lasting damages during fetal development. The fetuses may be especially vulnerable to EDCs because of their small size, rapid growth development, and limited ability to detoxify harmful substances [7]. Most EDCs are capable of easily crossing the placenta and enter the fetal bloodstream. In the last decades, studies regarding the transplacental transfer of EDCs and their determination in cord blood, serum, and maternal adipose tissues were performed [8–21]. When fat is mobilized during pregnancy or lactation, either the fetus or the newborn baby may be exposed to substantially high EDC levels [22]. The main effect of their prenatal exposure is associated to a decrease in gestational age, preterm birth, lower birth weight, growth retardation, and altered psychomotor and cognitive functions [23–28]. All cited literature clearly demonstrates how EDCs accumulate in placenta or are released in maternal blood, affecting the normal development of the fetus. During the last few years, the study of a possible correlation between Sudden Infant Death Syndrome (SIDS) and Sudden Intrauterine Unexpected Death Syndrome (SIUDS) and EDCs exposure has become a major issue [29–32]. SIDS is the sudden and unexpected death of an apparently healthy infant, within the first year of life. The unexpected death of a fetus after the 25th week of pregnancy is called SIUDS, which has an incidence six-fold greater than SIDS. Despite these syndromes representing the most relevant death-cause in fetuses and newborns in developed countries, their triggers are still unknown. This is due to a lack of detailed post mortem studies and undetermined environmental cofactors. To the best of our knowledge, we found no studies regarding the determination of EDCs in fetal and newborn tissues. The aim of this study was to develop a rapid and sensitive method of investigating the presence of a group of EDCs in human fetal and newborn tissues collected from SIUDS and SIDS autopsy findings for risk assessment purposes and to evaluate the presence of other non-targeted compounds in the same samples. The analyses were performed in samples from fetuses died sine causa after the 25th gestational week and SIDS victims coming from an intensive agricultural area in northern Italy. The main requirements for the proper application of the National Italian Law number 31: "Regulations for diagnostic post mortem investigation in victims of SIDS and unexpected fetal death," which was recently approved (2 February, 2006) were addressed. This law imposes that all suspected cases of SIDS, and all fetuses deceased after the 25th week of gestation without any apparent cause must undergo an in-depth anatomo-pathologic examination. For each autopsy, when possible, portions of liver and brain were collected. Twenty-five specific target EDCs were selected according to the local environmental conditions, including OCPs, OPPs, and carbamates. The selection of the target compounds was made including a group of ubiquitous contaminants such as the OCPs. In addition, exposure to OCPs is well known for causing preterm birth and delayed brain development [24]. The selected OPPs and carbamates were added to the list because they are commonly utilized in the region of origin of the victims, an intensive agricultural area of apples and grapes cultivations with a massive use of these pesticides [33]. In the last few years, special attention was focused on human exposure to bisphenol A (BPA) (4′-dihydroxy-2,2-diphenylpropane), a widely used industrial plasticizer with well-known estrogenic properties. BPA can be found in paints, flame retardants, unsaturated polyester resins, plastic food packages, water containers, baby bottles, and food wrap. Numerous studies have confirmed leaching of BPA from food containers at detectable levels in a wide range packaged foods [34–36]. The hydrolysis of the ester bonds of BPA-based polymers and the non-polymerized monomer residues are responsible for its widespread contamination. Scientific literature reports that BPA cannot be considered a biologically important pollutant because it is metabolized and excreted relatively quickly. However, several works also confirm the passage of BPA across the placenta, where only a part of it is metabolized and excreted, suggesting a continuous exposure of the mother and fetus to BPA [37–39]. Recent studies demonstrated that BPA shows estrogenic activity at extremely low concentration [40, 41].

The method described in this work is based on GC-MS detection, and was developed and validated on animal liver and brain tissues. Detection was carried out using a GC-qMS with a quadrupole (q) analyzer. Fast-GC coupled to a time-of-flight (TOF) analyzer allows a good sensitivity in full-scan mode in a half-time of analysis compared with conventional GC-qMS. TOF technology shows intrinsically higher analytical information, valuable for possible non-targeted analysis and post-data processing. For these reasons, TOF-MS detection is a valuable tool in the search of contaminants that were not included in our list.

Finally, to assess the applicability, this method was applied to the analyses of post mortem tissues collected during autopsies in the period 2011–2013.

Materials and methods

Chemicals and reagents

A 2000 µg/mL mixture of 20 organochlorine pesticides in toluene: *n*-hexane (50:50, vol/vol) (1) Aldrin (2) α-BHC (3) β-BHC (4) Lindane (5) δ-BHC (6) α-Chlordane (7) γ-Chlordane (8) 4,4'-DDD (9) 4,4''-DDE (10) 4,4′-DDT (11) Dieldrin (12) α-Endosulfan (13) β-Endosulfan (14) Endosulfan sulfate (15) Endrin (16) Endrin aldehyde (17) Endrin ketone (18) Heptachlor (19) Heptachlor epoxide isomer B (20) Methoxychlor) was purchased from Sigma-

Aldrich, EPA CLP Mix (Milan, Italy). A diluted mixture of OCPs in toluene:*n*-hexane (50:50, vol/vol) was prepared at a concentration of 200 μg/mL. Chlorpyrifos, chlorfenvinfos, captan, boscalid, and bisphenol A were purchased from Sigma-Aldrich. Stock solutions were prepared in *n*-hexane at a concentration of 200 μg/mL. Nine isotopically labeled internal standards (ISTD) were used for method validation. Chlorpyrifos D10, chlorfenvinfos D10, captan D6, alpha-endosulfan D4, beta-endosulfan D4, *p,p′*-DDE D8, *p,p′*-DDT D8, methoxychlor D14, bisphenol A D16 were purchased from Dr. Ehrenstorfer,(Lab Service Analytica, Bologna, Italy). A standard mixture containing all compounds (25 specific EDCs and nine ISTD) was prepared by

appropriate dilution and stored at 4 °C. All solvents used (dichloromethane, *n*-hexane) were pesticide GC-grade, supplied by VWR International (Milan, Italy). Solid phase extraction (SPE) cartridges DSC-C18 (500 mg/6 mL) were supplied by Supelco (Milan, Italy).

Instrumentation

Chromatographic analyses of EDCs were performed with an Agilent Technologies GC 6890 N equipped with a single quadrupole mass spectrometer 5975C TAD/MS operating in electron ionization (EI) mode and an Agilent 7683B autosampler (Agilent Technologies, Palo Alto, CA,

Table 1 Retention times, CAS number, molecular weight, and target ions for the GC-MS analysis of the selected compounds

Compound (order elution)	t_r (min)	CAS number	Molecular weight	Quantifier (bold) and qualifier ions (*m/z*)
α-BHC	7.88	319-84-6	288	**181**, 183, 219
β-BHC	8.53	319-85-7	288	**181**, 183, 219
γ-BHC	8.65	58-89-9	288	**181**, 183, 219
δ-BHC	9.28	319- 86-8	288	**181**, 183, 219
Heptachlor	10.65	76-44-8	373	100, 272, **337**
Aldrin	11.79	309-00-2	362	**263**, 265, 293
Chlorpyrifos D10	11.90	285138-81-0	360	200, 260, **324**
Chlorpyrifos	12.04	2921-88-2	350	258, 199, **314**
Heptachlor epoxide	13.30	1024-57-3	386	**353**, 355, 351
Captan D6	13.54	-	306	84, **154**, 117
Chlorfenvinfos D10	13.54	-	369	269, 271, **333**
Captan	13.64	133-06-2	300	79, 149, **264**
Chlorfenvinfos	13.69	470-90-6	359	267, 269, **323**
γ-Chlordane	14.36	57-74-9	406	**373**, 375, 377
α-Endosulfan D4	14.83	203645-57-2	411	172, **237**, 343
α-Endosulfan	14.92	959-98-8	407	**237**, 277, 339
α-Chlordane	15.01	5103-71-9	406	**373**, 375, 377
Bisphenol A D16	15.91	96210-87-6	244	223, **224**, 242
Bisphenol A	16.12	80-05-7	228	**213**, 228, 119
p,p′-DDE D8	16.28	93952-19-2	326	**326**, 184, 254
Dieldrin	16.32	60-57-1	378	277, **79**, 263
p,p′-DDE	16.37	72-55-9	316	318, **246**, 176
Endrin	17.44	72-20-8	378	**263**, 265, 81
β-Endosulfan D4	17.77	203716-99-8	411	**237**, 164, 343
β-Endosulfan	17.83	33213-65-9	407	195, **237**, 207
p,p′-DDD	18.23	72-54-8	318	**235**, 237, 165
Endrin aldehyde	18.46	7421-93-4	378	**67**, 250, 345
Endosulfan sulphate	19.08	1031-07-8	420	**274**, 272, 387
p,p′-DDT D8	19.17	93952-18-2	362	**243**, 245, 173
p,p′-DDT	19.22	50-29-3	355	**235**, 237, 165
Endrin ketone	20.05	53494-70-5	378	315, 67, **317**
Methoxychlor D14	20.30	-	359	**241**, 242, 288
Methoxychlor	20.37	72-43-5	344	**227**, 228, 214
Boscalid	22.74	188425-85-6	343	112, **140**, 342

USA). Analytes were separated using an HP-5MS (Agilent J&W GC columns; Folsom, CA, USA), 30.0 m×0.25 mm i.d., containing 5 % phenyl-methylsiloxane, with a phase thickness of 0.25 μm. The GC oven temperature was programmed as follows: initial temperature 80 °C, held for 1 min, ramped at 30 °C/min to 180 °C, ramped at 3 °C/min to 225 °C, held for 4 min, ramped at 20 °C/min to 300 °C, held for 4.08 min, (total acquisition time: 25 min). The injector was set at 250 °C in splitless mode. The injection volume was 1 μL. A 7.5-min solvent delay was set. Helium was used as carried gas at 1 mL/min, constant flow (SOL S.p.A, Ancona, Italy). The ion source and transfer-line were kept at 290 °C and 300 °C, respectively. The data acquisition was carried out in selected-ion monitoring (SIM) mode, with a 10-group program for the selection of target ions on different time windows defined by the corresponding retention times. Three ions for each analyte were selected according to the mass spectra recorded in the full-scan mode as well as by comparison with the National Institutes of Standards and Technologies (NIST) library. Table 1 shows the retention time, CAS number, molecular weigh, and the target ions of all analytes.

Fast screening of non-targeted compounds were performed using a DANI Master GC coupled to a TOFMS DANI Master TOF operating in EI mode and a DANI Master AS autosampler (DANI Instrument S.p.A, Cologno Monzese, Italy). Fast-GC provides a significant decrease in the analysis time. When a positive identification is required through a full-scan mass spectrometric detection using electron ionization, TOFMS is the only technology that provides the highest sensitivity. In fact, differently from a quadrupole analyzer, time-of-flight collects all the ions generated during the acquisition process. DANI Master TOF Plus MS detector performs a very fast acquisition rate (up to 1000 spectra/s) and a wide dynamic range (>105). Analytes were separated using a Rxi-5 ms (Restek Corporation, Bellefonte, PA, USA), 10.0 m×0.10 mm i.d., with a phase thickness of 0.10 μm. GC oven temperature was programmed as follows: initial temperature 80 °C, ramped at 30 °C/min to 180 °C, ramped at 20 °C/min to 205 °C held for 2 min, ramped at 20 °C/min to 300 °C held for 2 min, (total acquisition time: 15 min). The injector was set at 250 °C in splitless mode. The injection volume

Table 2 Method quality parameters obtained in real liver matrix

Compound	ISTDs	Method LOD (ng/g)	Method LOQ (ng/g)	Linear regression	R^2
α-BHC	α-Endosulfan D4	0.2	0.4	y =6.71x+22.15	0.9997
β-BHC	α-Endosulfan D4	0.2	0.4	y =3.75x − 35.88	0.9932
γ-BHC	α-Endosulfan D4	0.2	0.4	y=2.89x+13.32	0.9995
δ-BHC	α-Endosulfan D4	0.2	0.4	y=12.30x+2.70	0.9957
Heptachlor	α-Endosulfan D4	0.3	0.8	y=914.30x+12.86	0.9981
Aldrin	α-Endosulfan D4	0.3	0.8	y=423.40x+8265.00	0.9989
Chlorpyrifos	Chlorpyrifos D10	0.2	0.4	y=17.89x+1.73	0.9954
Heptachlor epoxide	β-Endosulfan D4	0.3	0.8	y=5.61x − 21.60	0.9995
Captan	Captan D6	0.8	1.6	y=54.42x+56.24	0.9879
Chlorfenvinfos	Chlorfenvinfos D10	0.4	1.6	y=684.01x+41.02	0.9996
γ-Chlordane	β-Endosulfan D4	0.2	0.4	y=1,9154x+0,613	0.9999
α-Endosulfan	α-Endosulfan D4	2.0	2.8	y=931.01x − 4396.00	0.9946
α-Chlordane	β-Endosulfan D4	0.2	0.4	y=7.36x − 42.57	0.9954
Bisphenol A	Bisphenol A D13	0.2	4.0	y=2.98x+12.41	0.9997
Dieldrin	p,p-DDE D8	2.0	4.0	y=6.43x − 14.82	0.9992
p,p′-DDE	p,p-DDE D8	0.2	0.4	y=32.99x − 6404.00	0.9978
Endrin	p,p-DDE D8	2.0	4.0	y=2.46x − 77.65	0.9961
β-Endosulfan	β-Endosulfan D4	2.0	4.0	y=3.84x+16.05	0.9991
p,p′-DDD	p,p-DDT D8	0.2	0.4	y=47.79x+35.43	0.9988
Endrin aldehyde	p,p-DDT D8	1.2	4.0	y=0.3542x+0.024	0.9972
Endosulfan sulphate	p,p-DDT D8	1.2	4.0	y=156.70x+9810.00	0.9931
p,p′-DDT	p,p-DDT D8	0.1	0.4	y=7.89x+1.09	0.9992
Endrin ketone	p,p-DDT D8	0.4	2.0	y=24.43x+104.50	0.9914
Methoxychlor	Methoxychlor D14	0.8	2.0	y=30.45x+19.65	0.9998
Boscalid	Captan D6	1.2	4.0	y=29.36x+14.32	0.9988

was 1 μL. The solvent delay of 3.5 min was selected. Helium was used as carried gas at 0.5 mL/min, constant flow (SOL S.p.A, Ancona, Italy). The ion source and transfer-line were kept at 290 °C and 200 °C, respectively. The data acquisition was carried out in full-scan mode in a range of 50–500 u.

Sample collection and storage

Five liver (3 from SIDS and 2 from SIUDS) and five brain (3 from SIDS and 2 from SIUDS) post mortem sample cases were collected during autopsies at the Department of Surgical, Reconstructive, and Diagnostic Sciences (University of Milan) according to the International Standardized Autopsy Protocol (ISAP) of the Global Strategy Task Force of SIDS International, the International Stillbirth Alliance, and the national Italian Law number 31, 2 February, 2006, Regulations for Diagnostic post mortem Investigation in Victims of Sudden Infant Death Syndrome (SIDS) and Unexpected Fetal Death. After collection, the tissue samples were preserved before analyses at –20 °C.

Extraction method

EDCs were extracted from tissue samples according to the liquid-solid method proposed by Fernandes and co-workers [42]. In our work, this method, originally developed for fat tissues, was applied for the first time to liver and brain tissues. Aliquots of 500 mg of matrix were fortified with 5 μL of standard solution in acetone containing the selected compounds and internal standards (only ISTDs in human samples) in order to obtain the following fortification levels in the tissue samples: 8.0, 120.0, and 8000 ng/g. For ISTDs, the concentration was of 150 ng/g. These values were selected on the basis of a low, medium, and high concentration with respect to method LOQs. Acetone was chosen as a solvent because of its miscibility in water and volatility. After 2 h, the acetone was evaporated and the analytes were extracted after homogenization with a Teflon tip using 2 mL of *n*-hexane, leaving a dense, rich supernatant. After extraction, an SPE clean-up procedure was added. One mL of the supernatant was transferred to an SPE cartridge placed on a 12-port Visiprep SPE vacuum manifold (Supelco, Bellefonte, PA, USA), previously conditioned with 4 mL of *n*-hexane. Before purification, the

Table 3 Method quality parameters obtained in real brain matrix

Compound	ISTDs	Method LOD (ng/g)	Method LOQ (ng/g)	Linear regression	R^2
α-BHC	α-Endosulfan D4	0.2	0.4	y=14.603x+11.61	0.9961
β-BHC	α-Endosulfan D4	0.2	0.4	y=15.309x+39.38	0.9973
γ-BHC	α-Endosulfan D4	0.2	0.4	y=31.219x+7.61	0.9987
δ-BHC	α-Endosulfan D4	0.2	0.4	y=22.478x+8.24	0.9966
Heptachlor	α-Endosulfan D4	0.3	0.8	y=3.735x − 9.47	0.9976
Aldrin	α-Endosulfan D4	0.3	0.8	y=2.957x − 6.80	0.9966
Chlorpyrifos	Chlorpyrifos D10	0.2	0.4	y=2.860x − 4.31	0.9974
Heptachlor epoxide	β-Endosulfan D4	0.3	0.8	y=13.585x − 7.61	0.9996
Captan	Captan D6	0.8	1.6	y=19.652x+14.76	0.9968
Chlorfenvinfos	Chlorfenvinfos D10	0.4	1.6	y=29.302x+24.84	0.9968
γ-Chlordane	β-Endosulfan D4	0.2	0.4	y=1517x − 2365.00	0.9983
α-Endosulfan	α-Endosulfan D4	2.0	2.8	y=2.496x − 6.24	0.9984
α-Chlordane	β-Endosulfan D4	0.2	0.4	y=3219x − 1063.00	0.9967
Bisphenol A	Bisphenol A D13	0.2	4.0	y=7.497x+5.23	0.9996
Dieldrin	p,p-DDE D8	2.0	4.0	y=4.228x − 6.50	0.9962
p,p′-DDE	p,p-DDE D8	0.2	0.4	y=2.679x+2.37	0.9999
Endrin	p,p-DDE D8	2.0	4.0	y=1.522x − 0.73	0.9961
β-Endosulfan	β-Endosulfan D4	2.0	4.0	y=3.646x − 7.43	0.9977
p,p′-DDD	p,p-DDT D8	0.2	0.4	y=7.711x − 3.74	0.9995
Endrin aldehyde	p,p-DDT D8	1.2	4.0	y=14.644x − 6.77	0.9978
Endosulfan sulphate	p,p-DDT D8	1.2	4.0	y=4.955x − 0.81	0.9964
p,p′-DDT	p,p-DDT D8	0.1	0.4	y=3.798x − 7.27	0.9966
Endrin ketone	p,p-DDT D8	0.4	2.0	y=1.687x − 0.86	0.9969
Methoxychlor	Methoxychlor D14	0.8	2.0	y=1.026x+2.00	0.9972
Boscalid	Captan D6	1.2	4.0	y=17.998x+3.55	0.9984

cartridge was dried under vacuum for 15 min. In this way, most of the matrix impurities were retained in the cartridge while the compounds of interest were eluted with the hexane. The clean-up procedure was completed with 1 mL of *n*-hexane followed by 1 mL of dichloromethane. Prior to injection, the final SPE extract in *n*-hexane:dichloromethane (1:1; vol/vol) was evaporated with a stream of nitrogen to a volume of 200 μL and analyzed by GC-MS.

Results and discussion

For ethical reasons and for the scarce availability of real samples, all validation experiments were carried out using the corresponding animal tissues (swine liver and lamb brain). Differences in the matrix composition compared with the corresponding human tissues are negligible in terms of extraction efficiency and other interactions that may influence the quality of the analytical data, as demonstrated in most dedicated literature [43–45]. Animal blanks underwent the entire

Table 4 Recoveries for each analyte at fortification levels in liver matrix: 8.0, 120.0, and 8000 ng/g

Compound	Rec±RSD 8.0 ng/g	Rec±RSD 120.0 ng/g	Rec±RSD 8000.0 ng/g
α-BHC	97±6	102±2	100±5
β-BHC	94±7	97±3	101±9
γ-BHC	98±3	102±2	99±7
δ-BHC	99±4	106±1	97±7
Heptachlor	92±9	107±1	99±2
Aldrin	93±4	107±3	101±6
Chlorpyrifos	99±2	98±4	97±5
Heptachlor epoxide	98±6	96±2	100±4
Captan	89±7	93±3	98±4
Chlorfenvinfos	96±4	107±3	99±1
γ-Chlordane	97±3	101±1	97±6
α-Endosulfan	99±4	94±2	99±2
α-Chlordane	98±3	105±1	104±8
Bisphenol A	101±4	99±1	103±0
Dieldrin	96±6	93±1	100±0
p,p′-DDE	94±2	105±2	99±2
Endrin	98±3	103±2	99±3
β-Endosulfan	95±7	99±3	98±8
p,p′-DDD	97±3	97±4	97±2
Endrin aldehyde	86±5	94±3	102±3
Endosulfan sulphate	97±4	99±4	101±4
p,p′-DDT	99±2	97±2	106±3
Endrin ketone	100±4	94±4	99±6
Methoxychlor	93±7	99±3	104±5
Boscalid	87±6	90±4	99±4

Table 5 Recoveries for each analyte at fortification levels in brain matrix: 8.0, 120.0, and 8000 ng/g

Compound	Rec±RSD 8.0 ng/g	Rec±RSD 120.0 ng/g	Rec±RSD 8000.0 ng/g
α-BHC	94±8	99±6	100±2
β-BHC	93±7	97±8	99±1
γ-BHC	94±10	98±5	102±2
δ-BHC	93±4	89±6	99±7
Heptachlor	91±9	101±1	99±2
Aldrin	98±2	99±3	98±6
Chlorpyrifos	97±6	103±4	98±4
Heptachlor epoxide	91±3	94±6	100±6
Captan	9244±6	96±7	99±4
Chlorfenvinfos	95±6	97±39	97±5
γ-Chlordane	96±5	99±4	100±3
α-Endosulfan	83±2	94±7	99±4
α-Chlordane	93±9	97±7	102±5
Bisphenol A	92±4	95±4	98±6
Dieldrin	89±11	93±9	106±3
p,p′-DDE	94±6	99±6	99±8
Endrin	97±9	88±4	96±4
β-Endosulfan	91±3	96±6	98±5
p,p′-DDD	96±4	85±8	100±6
Endrin aldehyde	91±3	101±6	99±5
Endosulfan sulphate	96±6	99±4	102±1
p,p′-DDT	97±6	97±2	99±4
Endrin ketone	93±8	93±1	102±1
Methoxychlor	93±1	96±4	99±4
Boscalid	94±4	94±6	97±8

analytical procedure prior to fortification, in order to assess the absence of target compounds. This procedure was applied to

Table 6 Target compounds in liver and brain tissues from fetal and infant autopsy cases

Compound	Case 1[a] (ng/g) (liver)	Case 2[b] (ng/g) (brain)	Case 3[c] (ng/g) (brain)	Case 4[d] (ng/g) (brain)
Heptachlor	n.d	7.4	n.d.	n.d.
γ-Chlordane	n.d	6.6	25	10.2
Endrin	63.5	n.d.	n.d.	n.d.
α-Chlordane	n.d.	6.9	34	11.3
p,p'-DDE	3.5	n.d	n.d.	n.d.

n.d.: not detected.

[a] Female. SIUDS, 40 wk of pregnancy.

[b] Male. SIDS, 1 mo.

[c] Male. SIDS, newborn died 1 h after birth.

[d] Female. SIUDS, 35 wk of pregnancy.

each liver and brain sample used to validate the method. Tables 2 and 3 show the method quality parameters obtained in real matrices. The method linearity was evaluated using five concentrations of analytes, starting from method LOQ to more than four orders of magnitude: 0.4, 4.0, 40.0, 4000, and 8000 ng/g in liver matrix (Table 2) and 4.0, 20.0, 400.0, 2000.0, and 4000.0 ng/g in brain matrix (Table 3). Each concentration was injected three times. The calibration curves for all investigated EDCs were linear, with coefficients of correlation (R^2) spanning from 0.9879 to 0.9999 in liver matrix and from 0.9961 to 0.9995 in brain matrix. Precision was assessed in terms of intra-day ($n = 5$) and inter-day repeatability (5 d, $n = 25$), calculating the relative standard deviation by injecting a 100 ng/mL standard solution. The intra-day and inter-day repeatability ranged from 1 % to 8 % and 3 % to 12 % of relative standard deviation (RSD%), respectively. The analytical method proved to be sensitive enough to analyze and detect the very low amounts expected of the selected compounds possibly present in real autopsy findings. Methods LODs and LOQs were evaluated using GC-MS at

a signal-to-noise ratio of 3 (S/N=3) and 10 (S/N=10), respectively. Method detection limits were in the range of 0.16-1.6 ng/g and method quantification limits spanned from 0.4 ng/g–4.0 ng/g in both matrices. Finally, method accuracy was evaluated with recovery studies after spiking noncontaminated animal tissues at three different concentration levels. Table 4 and 5 list the calculated recoveries for each analyte at the fortification levels of 8.0, 120.0, and 8000 ng/g in liver and brain matrices, respectively. As it can be seen, the recoveries were higher than 80 % for most of the selected EDCs.

The proposed method was applied to the analysis of fetal and newborn liver and brain tissues collected during autopsies of four SIDS and six SIUDS cases.

Among the target analytes, five OCPs in four autopsy cases were detected (Table 6). The results obtained showed that the fetal liver sample was positive to two selected EDCs, namely p,p'-DDE and endrin, which were detected at a concentration of 3.5 ng/g and 63.5 ng/g, respectively. Three brain samples were positive to the other three selected EDCs: heptachlor, γ-
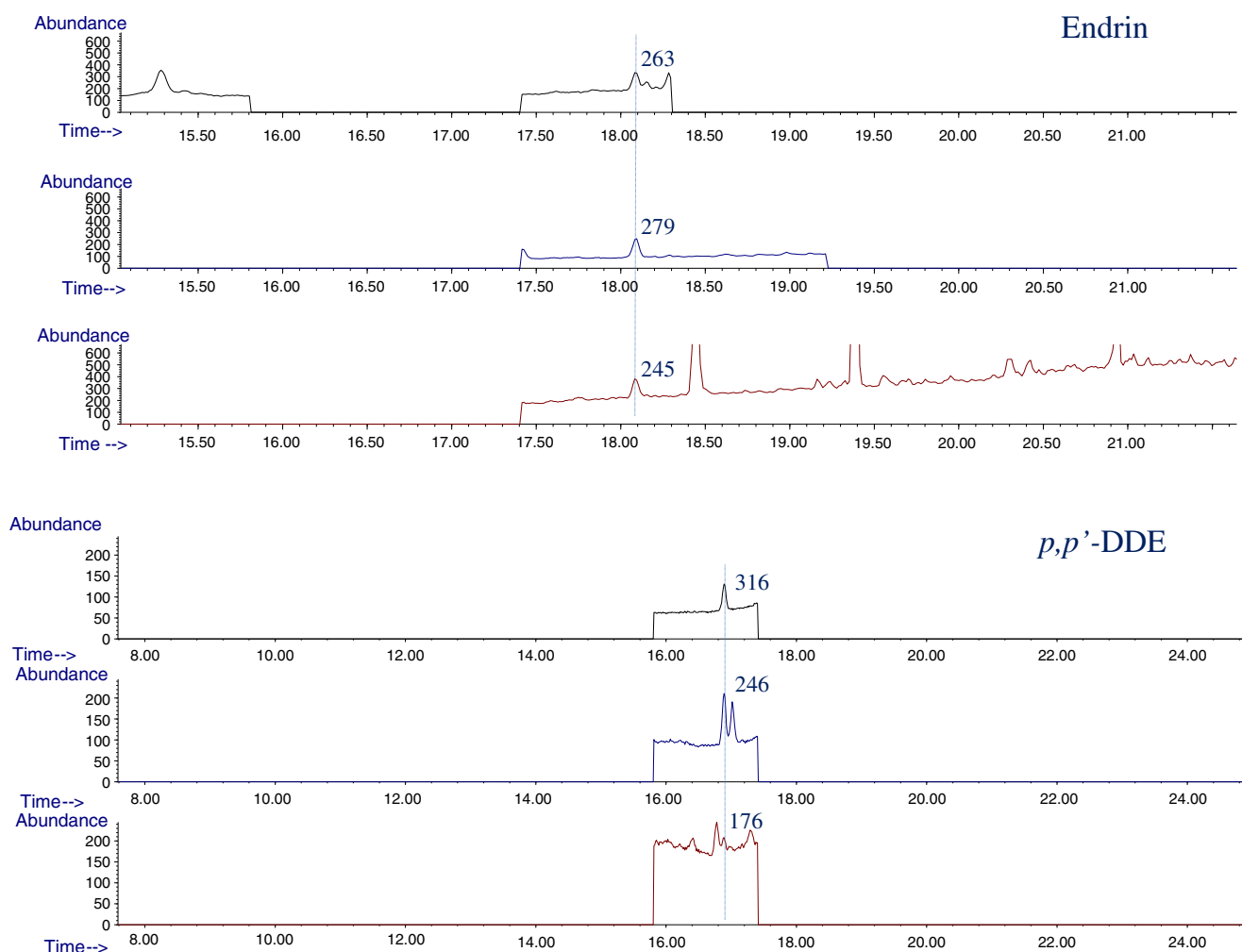

Fig. 1 Extracted ion chromatograms of Endrin and p,p'-DDE from the analysis of a fetal liver sample

chlordane, and α-chlordane ranging from 6.6–34 ng/g. As shown in the table, the four positive samples were distributed as follows: Case a, Female, SIUDS, 40 wk of pregnancy; Case b, Male, SIDS, 1 mo; Case c, Male, SIDS, newborn died 1 h after birth; Case d, Female, SIUDS, 35 wk of pregnancy.

Figure 1. shows the extracted ion chromatograms of a liver real sample containing p,p′-DDE and endrin. The identification was based on retention times and three characteristic ions for each compound and their relative abundance. This result is in accordance with previous literature data that report the presence of these specific target compounds in other human tissues, such as lung, muscle, and kidney [46–48]. OCPs have been used extensively in agriculture and although most of them were banned since 1970 and are no longer used, they are still present in the environment and may be routinely detected in surface water, fish, wildlife, food, and even in humans. The presence of p,p′-DDE in human tissues [49, 50] may be related to a direct exposure to this compound present in the environment. Finally, even though the

contaminants were only detected in the low ppb range, their presence is significant considering the high degree of toxicity and the vulnerability of the small, immature body. The detection of heptachlor, γ-chlordane, and α-chlordane in brain samples are in good correlation with in-depth anatomo-pathological examination of both the autonomic nervous system and of the cardiac conduction system, according to the application of the guidelines provided by the Italian law n.31/2006 Regulations for Diagnostic Post Mortem Investigation in Victims of Sudden Infant Death Syndrome and Unexpected Fetal Death. Developmental alterations of many vital centers of the brainstem were observed in three out of four cases. Noteworthy was the association of the presence of toxic compounds in the cerebral cortex with the heterozygote genotype S/L of the serotonin transporter gene, detected in two out the four cases. The S (short) allele is in fact more prevalent in the control population, the L (long) allele, on the contrary, is a rare finding, although the L/L and S/L genotypes are specifically observed in several cases of SIDS.

Fig. 2 (a) FastGC-TOFMS full scan chromatogram of a brain sample extract; (b) extracted ion chromatogram of m/z 256; (c) mass spectrum related to the peak at 8 min of retention time. NIST library identified the mass spectrum as pentachlorobiphenyl. *Pentachlorobiphenyl peak
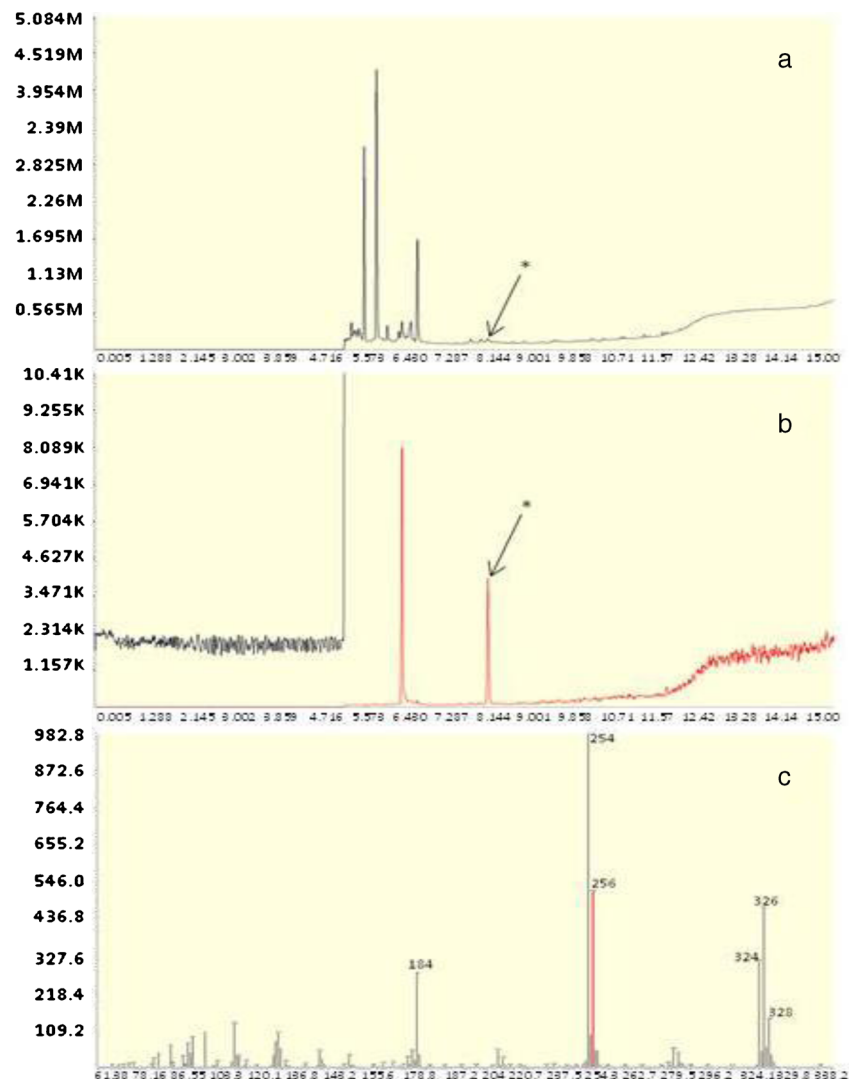
Time-of-flight experiments

All extracted samples were analyzed in full scan mode using fast GC-TOFMS instrumentation in search of non-targeted compounds. As qualitative only evaluation and using a fast GC separation, this additional procedure was rapid and gave the chance to examine the sample from a different perspective. The samples were carefully examined using a proprietary DANI Master LAB data processing equipped with a NIST 2011 mass spectra library ver. 2.0 that includes an AMDIS deconvolution algorithm for compound identification in the presence of numerous matrix interfering signals. Of course, as expected, the presence of abundant matrix substances in low mass resolution conditions made the detection of contaminants particularly challenging. Fortunately, the software provides an automated background subtraction for each detectable chromatographic peak for a cleaner, faster, and more effective library searching. As a result of this procedure, in one of the new brain samples (Case 5[e]), from a SIUDS victim, the presence of pentachlorobiphenyl was clearly highlighted (Fig. 2). The figure shows the full scan chromatogram (Fig. 2a), the m/z 256 ion profile (Fig. 2b), and the mass spectrum (Fig. 2c). Ion at m/z 256 is characteristic of pentachlorobiphenyl and other polychlorobiphenyls (PCBs). Identification quality parameters were: match 721, rev. match 730, probability 12.5 %, ranking #1. These numbers were in good accordance with the noisy background, typical of a real-sample identification. The spectrum was easily recognizable for the typical chlorine isotopic cluster even though slight differences in the ion abundances can be observed. This result was somehow expected, considering the ubiquitous presence of PCBs in the environment, but it strengthens the use of TOF as a good scouting tool. Thanks to the high acquisition rate of this specific instrument (5–20 full spectra/s), future experiments may include GCxGC separations for additional specificity and reduced influence of the matrix.

Conclusions

To the best of our knowledge, the method proposed has been applied for the first time to fetal and newborn liver and brain tissues, collected post mortem during autopsies. The use of a double GC-MS system employing quadrupole and time-of-flight analyzers allows accurate quantitation of target analytes and opens the possibility to the detection of non-targeted contaminants. The extraction of 25 EDCs from the samples is simple and rapid and provides a good linearity range and repeatability, and high extraction efficiency. The selection of the EDCs was limited to those commonly used in a restricted agricultural area. However, the method proposed can be easily extended to other compounds for a more comprehensive study, and can be applied to an epidemiologic investigation to correlate the exposure to EDCs and SIDS and SIUDS for risk assessment purposes. Furthermore, the limited number of cases studied so far already demonstrates the presence of a few target and non-targeted compounds, and shows an interesting correlation with anatomo-pathologic observations. More studies, involving a larger selection of analytes and a higher number of cases, are needed to address whether maternal exposure to EDCs can lead to adverse health effects on fetuses and babies, and if this exposure can be correlated to SIDS and SIUDS.

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