Anal Bioanal Chem (2015) 407:2463–2472
DOI 10.1007/s00216-015-8492-x

RESEARCH PAPER

Occurrence of specific environmental risk factors in brain tissues of sudden infant death and sudden intrauterine unexpected death victims assessed with gas chromatography–tandem mass spectrometry

Veronica Termopoli · Giorgio Famiglini · Pierangela Palma · Laura Magrini · Achille Cappiello

Received: 10 December 2014 / Revised: 9 January 2015 / Accepted: 13 January 2015 / Published online: 10 February 2015

Abstract Sudden infant death syndrome (SIDS) and sudden intrauterine unexpected death syndrome (SIUDS) are an unresolved teaser in the social–medical and health setting of modern medicine and are the result of multifactorial interactions. Recently, prenatal exposure to environmental contaminants has been associated with negative pregnancy outcomes, and verification of their presence in fetal and newborn tissues is of crucial importance. A gas chromatography–tandem mass spectrometry (MS/MS) method, using a triple quadrupole analyzer, is proposed to assess the presence of 20 organochlorine pesticides, two organophosphate pesticides, one carbamate (boscalid), and a phenol (bisphenol A) in human brain tissues. Samples were collected during autopsies of infants and fetuses that died suddenly without any evident cause. The method involves a liquid–solid extraction using *n*-hexane as the extraction solvent. The extracts were purified with Florisil cartridges prior to the final determination. Recovery experiments using lamb brain spiked at three different concentrations in the range of 1–50 ng g^{-1} were performed, with recoveries ranging from 79 to 106 %. Intraday and interday repeatability were evaluated, and relative standard deviations lower than 10 % and 18 %, respectively, were obtained. The selectivity and sensitivity achieved in multiple reaction monitoring mode allowed us to achieve quantification and confirmation in a real matrix at levels as low as 0.2–0.6 ng g^{-1}. Two MS/MS transitions were acquired for each analyte, using the Q/q ratio as the confirmatory parameter. This method was applied to the analysis of 14 cerebral cortex samples (ten SIUDS and four SIDS cases), and confirmed the presence of several selected compounds.

Keywords Environmental risk factors · Gas chromatography–tandem mass spectrometry · Triple quadrupole · Sudden intrauterine unexpected death syndrome · Sudden infant death syndrome

Introduction

Sudden intrauterine unexpected death syndrome (SIUDS) and sudden infant death syndrome (SIDS) are still main medical and social challenges in developed countries. SIUDS is defined as the unexpected death of an apparently healthy fetus after the 25th week of pregnancy in the absence of infections or malformations. SIDS is the sudden death of an infant younger than 1 year of age, and which remains unexplained even after an investigation of the circumstances of death. This investigation includes a complete autopsy, an examination of the death scene, and a review of the infant's clinical history. Several factors play important roles in the occurrence of these deaths, including genetic alterations and exposure to certain risk factors, such as maternal smoking, alcohol abuse, and drug abuse. The exposure to these risk factors has been related to an increase in the risk of SIDS and SIUDS, yielding

Electronic supplementary material The online version of this article (doi:10.1007/s00216-015-8492-x) contains supplementary material, which is available to authorized users.

V. Termopoli (✉) · G. Famiglini · P. Palma · L. Magrini · A. Cappiello
LC-MS Laboratory, Department of Earth, Life and Environmental Sciences, University of Urbino, Piazza Rinascimento 6, 61029 Urbino, Italy
e-mail: veronica.termopoli@uniurb.it

phenotypes prone to these syndromes [1–8]. However, in many cases the exposure to those risk factors has not been proved, and new studies have been directed to the adverse effects of environmental pollution. Most of the studies have been focused on specific classes of compounds, such as organochlorine pesticides (OCPs), polybrominated diphenyl ethers, and polychlorinated biphenyls, because they are highly resistant to degradation, are lipophilic, and tend to bioaccumulate in adipose tissues and be biomagnified in the food chain. Although their use and production have been banned or restricted by the Stockholm Convention [9], these compounds are ubiquitously dispersed in the environment. During pregnancy, when fat is mobilized, the accumulated chemicals can easily cross the placenta and enter the fetal bloodstream. Once there, they can lead to several pregnancy outcomes, such as miscarriage, preeclampsia, reduced birth weight, growth retardation, and psychomotor and cognitive dysfunctions [10–16]. As a result, many current efforts are focused on the study of transplacental transfers of these classes of compounds, and on their determination in umbilical cord blood, serum, and maternal adipose tissues, to understand better the dynamics of in utero exposure [17–23]. Chemicals can also pass from breast milk to newborns, exposing them to high concentrations [24]. Some compounds, such as organophosphate pesticides (OPPs), boscalid, and bisphenol A (BPA) are considered nonpersistent in the environment, but nevertheless they are used and produced in such great quantities that the population is continuously exposed to them, although at a low concentration [25]. OPPs and boscalid are the most widely used insecticides available today and are used in agriculture, homes, gardens, and veterinary practice. BPA is an industrial chemical used to make hard clear plastic known as polycarbonate, which is used in many consumer products, including reusable bottles and baby bottles. It is a pseudopersistent chemical, which despite its short half-life is ubiquitous in the environment, where it is continuously released. Several recent studies have associated adverse health outcomes in newborns with maternal exposure to OPPs and BPA during gestation, including abnormal reflexes, reduced cognitive abilities, and attention problems [26–30]. Moreover, these compounds are reported to cross the placenta, posing a threat during brain development of the fetus [31, 32]. Although many studies on transplacental transfer have been performed, very few studies on the detection of environmental contaminants in fetal tissues have been reported [33–38]. In addition, none of these studies concern the determination of pollutants in fetal and newborn brain tissues. In light of all these factors, the assessment of fetal exposures, together with the determination of environmental contaminants in brain tissues collected from SIUDS and SIDS victims, is key to help identify a possible relationship with the abnormalities commonly found in the autonomic nervous system in both syndromes [39, 40]. All these articles describe methods based mainly on gas chromatography (GC)–mass spectrometry (MS), used in both ionization modes (electron ionization and negative chemical ionization) and GC–MS/MS. Our research group previously reported a GC–MS method for the extraction, identification, and quantification of 25 selected endocrine-disrupting compounds in liver and brain tissues from SIDS and SIUDS victims [41]. We demonstrated its ability to identify and quantify selected compounds, allowing their determination in several brain and liver tissues. Although GC–MS has been the most widely used technique for the determination of these specific compounds owing to its extreme reliability and simplicity, it may present deficiencies in ultratrace analysis of complex matrices such as biological tissues.

Therefore, owing to the very low amount of these environmental contaminants and the complexity of the matrix, it is necessary to improve further both sensitivity and selectivity. In view of the aforementioned facts, a new analytical method was developed using a GC–MS/MS instrument with a triple quadrupole (QqQ) analyzer. The use of GC–MS/MS rather than GC–MS allowed us to obtain approximately a tenfold improvement of the limits of detection (LODs). The method allowed us to improve selectivity and sensitivity, and to better confirm the actual presence of 20 OCPs, two OPPs, BPA, and boscalid in cerebral cortex of human fetal and newborn SIUDS and SIUD victims. Two OPPs, chlorpyrifos and chlorfenvinfos, and boscalid were chosen according to the use of pesticides in common Italian and European agricultural crops, such as peaches, oranges, and grapes [42–44]. We decided to include a large number of OCPs since they are the most studied compounds in maternal tissues and their significant toxicity is well documented in the literature [17, 20–23]. The use of a QqQ analyzer leads to adequate precursor and product ion selection, and allows one to increase the specificity and to minimize or eliminate matrix interferences. The use of multiple reaction monitoring (MRM) mode offers greater sensitivity compared with selected ion monitoring, because the fragmentation reaction implies two different characteristics of the target compounds [45]. Thanks to these properties, the use of MRM also ,makes it possible to simplify the clean-up procedure.

All analyses were conduced in samples from fetuses that died after the 25th week of pregnancy and SIDS victims (from 1 h to 12 months of life) from three intensive agricultural areas in northern Italy. The entire method addressed the main requirements for the proper application of national Italian law number 31/2006, “Regulations for diagnostic postmortem investigation in victims of SIDS and unexpected fetal death,” which was approved on February 2, 2006 [46]. This Italian law states that all infants that died suddenly within the first year of life of suspected SIDS and all fetuses that died after the 25th gestational week without any apparent cause must undergo an in-depth anatomopathological examination, particularly

of the autonomic nervous system [47, 48]. For each autopsy, a portion of cerebral cortex (1 cm^3, approximately) was collected. For ethical reasons and because of the scarce availability of real samples, all validation experiments were conducted using lamb brain tissue. Finally, the method was satisfactorily applied to 14 cerebral cortex samples collected from SIUDS and SIDS victims in the 2012–2014 period.

Experimental

Chemicals and reagents

A mix of OCPs (certified reference material) containing α-BHC, β-BHC, γ-BHC, δ-BHC, aldrin, α-chlordane, γ-chlordane, *p,p′*-DDE, *p,p′*-DDT, *p,p′*-DDD, α-endosulfan, β-endosulfan, endosulfan sulfate, endrin aldehyde, endrin ketone, heptachlor, heptachlor epoxide, dieldrin, endrin, and methoxychlor in toluene–*n*-hexane (50:50, v/v) at a concentration of 2,000 ng μL^{-1} (Environmental Protection Agency Contract Laboratory Program OCP pesticide mix) was purchased from Sigma-Aldrich (Milan, Italy). Chlorpyrifos, chlorfenvinfos, and boscalid (purity greater than 99 %) were obtained from Fluka (Milan, Italy). Five isotopically labeled compounds used as internal standards—BPA-D_{16}, *p,p′*-DDE-D_8, *p,p′*-DDT-D_8, methoxychlor-D_{14}, and chlorfenvinfos-D_{10}—were supplied by Dr. Ehrenstorfer (Augsburg, Germany). All solvents used (*n*-hexane, acetone, and toluene) were pesticide residue GC-grade PESTANAL (purity greater than 99.8 %), supplied by Fluka (Milan, Italy). The chemical structures of the compounds investigated are shown in Fig. S1. The Supelclean ENVI-Florisil solid-phase extraction cleanup cartridges (200 mg/3 mL) were purchased from Phenomenex (Bologna, Italy).

Standard solutions

A diluted solution of OCPs in *n*-hexane–toluene (50:50, v/v) was prepared at a concentration of 100 ng μL^{-1}. Stock standard solutions of each internal standard were prepared in acetone at a concentration of 100 ng μL^{-1}. The working standard solutions containing all the compounds investigated were prepared by diluting the stock solutions in *n*-hexane. All the solutions were stored in brown glass vials at 4 °C.

Brain sample collection and storage

For ethical reasons and because of the scarce availability of control cases (fetuses or newborns that died of evident causes unrelated to environmental contamination), all validation experiments were conducted using lamb cerebral cortex. Differences in the matrix composition compared with the corresponding human tissue are negligible in terms of extraction efficiency and other interactions that may influence the quality of the analytical data, as demonstrated in the literature [47]. Lamb brain was purchased from a local supermarket, cut into 0.5-g samples, and stored at -20 °C.

Ten SIUDS cerebral cortex (25–41 weeks of pregnancy) and four SIDS cerebral cortex (2 days to 4 months of age) samples were collected during autopsies performed in several hospitals in northern Italy over a 2-year period (2012–2014) (Table 1). The autopsies were performed in all cases according to the International Standardized Autopsy Protocol of the Global Strategy Task Force of SIDS International [49] and the neuropathology protocol developed at the Department of Surgical, Reconstructive, and Diagnostic Sciences (University of Milan, Italy) [47, 48, 50]. Once in the laboratory, the specimens were accurately weighed in 0.5-g amounts, when possible, and were preserved at –20 °C before analyses. All available information about pregnancy, fetal development, delivery, exposure to environmental pollution, and familial situation was collected. For SIDS cases, information regarding death circumstances was evaluated as well.

Preparation of spiked samples

Samples of 0.5 g of frozen lamb brain were finely minced. When defrosted, each sample was spiked with a 2.5 μL of a standard solution of the selected compounds in acetone at three different concentrations—0.2, 2.0, and 10.0 ng μL^{-1}—to obtain the following fortified concentrations in the tissues: 1.0, 10.0, and 50.0 ng g^{-1}. Then, 3.7 μL of the 20 ng μL^{-1} working solution of the internal standards in acetone was added to each sample to obtain a final concentration of 150 ng g^{-1}. The spiked samples of brain were homogenized in a glass centrifuge tube, using a Teflon tip, for 5 min and

Table 1 Description of sudden intrauterine unexpected death syndrome (*SIUDS*) and sudden infant death syndrome (*SIDS*) cases

	Gender	Syndrome and age	Sample name
1	Female	SIUDS (35 weeks)	F2
2	Female	SIUDS (34 weeks and 5 days)	F3
3	Female	SIUDS (39 weeks)	F4
4	Male	SIDS (2 days)	N5
5	Male	SIUDS (40 weeks)	F6
6	Female	SIUDS (30 weeks and 1 day)	F7
7	Male	SIUDS (39 weeks)	F8
8	Female	SIUDS (39 weeks and 3 days)	F9
9	Female	SIUDS (38 weeks and 4 days)	F10
10	Male	SIUDS (34 weeks and 1 day)	F11
11	Female	SIDS (4 months)	N12
12	Female	SIUDS (29 weeks and 5 days)	F13
13	Male	SIDS (4 months)	N14
14	Female	SIDS (4 months)	N15

were left for 2 h at room temperature, prior to extraction, to allow solvent evaporation and better distribution of the selected compounds and internal standards throughout the matrix. The same procedure was applied to the human samples, which of course were spiked only with the internal standards.

Extraction method

The homogenate was extracted by adding 1.5 mL of *n*-hexane and vigorously shaken with a vortex mixer (Super Mixer, Continental Instrument, USA) for 10 min. The whole homogenate was collected and centrifuged at 3,500 rpm for 5 min. After centrifugation, the supernatant was purified through a Florisil cartridge, previously conditioned with 2 mL of *n*-hexane, and was collected in a glass vial. Then, the sample was concentrated to 200 μL under nitrogen. Finally, 1 μL of extract was injected into the GC–MS/MS system.

GC–MS/MS instrumentation

A GC system (Agilent 7890B, Agilent Technologies, Palo Alto, CA, USA) was coupled to a QqQ mass spectrometer (Agilent 7000A, Agilent Technologies, Palo Alto, CA, USA) operating in electron ionization mode. The GC separation was performed using a fused-silica HP-5MS capillary column (30 m × 0.25-mm inner diameter) with a film thickness of 0.25 mm (Agilent J&W GC column, Agilent Technologies, Folsom, CA, USA). The oven temperature was set as follows: 80 °C (1 min), up to 180 °C at 30 °C min^{-1}; up to 225 at 3 °C min^{-1} (held for 4 min), then up to 300 °C at 20 °C min^{-1} (held for 1.08 min). The injector was set at 250 °C, operating in splitless mode using helium (purity 99.9 %; Air Liquide, Milan, Italy) as the carrier gas at a constant flow rate of 1 mL min^{-1}. The interface and the ion source temperature were kept at 290 °C and 300 °C, respectively. The QqQ MS system operated in MRM mode using nitrogen (purity 99.9 %) as the collision gas at a flow rate of 1.5 mL min^{-1} in the collision cell. Helium was used as the quenching gas in the collision cell at a flow rate of 2.2 mL min^{-1}. The filament current was set at 35 μA. MS spectra (scan range *m*/*z* 50–550, scan time 200 ms, 6.1 cycles per second) and MS/MS spectra (scan range *m*/z 50–400, 4.2 cycles per second) were acquired for each analyte in order to select the best precursor and product ions for MRM experiments. The retention times, precursor and product ions, and optimized collision energy for each analyte are listed in Table 2. Data acquisition and analyses were performed using the Agilent MassHunter Workstation software package supplied by Agilent Technologies. The MassHunter Quantitative Analysis program was used to process the quantitative data obtained from calibration matrices and from real samples.

Results and discussion

MS/MS optimization

Optimization of the MS/MS method was performed for all selected compounds using standard solutions in *n*-hexane. The full-scan spectra were obtained by injecting a 100 pg μL^{-1} standard mixture. The precursor ions of every compound were selected as the most abundant peaks of each spectrum. Different collision energies (from 15 to 30 eV) were tested to perform the fragmentation. For each compound, the collision energies that yielded the two precursor–two product ion transitions with the best signal intensity were chosen to build the MRM acquisition method. When this was not possible, two transitions from one precursor ion were used. The most intense transition was chosen as a quantifier (Q) and the second most intense transition was chosen as a qualifier (q), using the ion ratio criteria established by Commission Decision 2002/657/EC [51]. At least two transitions per compound were selected, with the exception of internal standards, which required only one transition, normally the most intense one. The Q and q transitions selected for every compound are shown in Table 2. The optimized collision energies and *Q*/*q* ratios are also listed in Table 2. Average *Q*/*q* ratios, obtained from standard solutions at three concentrations (50, 100, 200 pg μL^{-1}) each injected in triplicate (n=9), were kept as "theoretical values" and are also shown in Table 2. Satisfactory relative standard deviation of 15 % or less was achieved for all the compounds.

The dwell time was also optimized in order to obtain a good chromatographic peak. This parameter gave the best results for all compounds in the range from 0.01 and 0.7 s.

Confirmation ratio

The *Q*/*q* ratio was used to confirm peak identity in the real and spiked samples. The experimental *Q*/*q* ratios, obtained from real samples, were compared with the theoretical ones. Confirmation of analytes detected in real samples was considered positive when the experimental *Q*/*q* ratio was within ±20 % of the average *Q*/*q* value calculated from the standards, as established in Commission Decision 2002/657/EC [51]. This decision was originally defined for the determination of organic contaminants in food samples, although it is being increasingly used for the confirmation of positive findings in other matrices [52, 53]. These ratios, together with the retention time, provided unequivocal identification of the selected compounds.

In the case of complex matrices, such as brain, the use of MS/MS mode is very important to notably increase sensitivity and selectivity, allowing one to discriminate between many interfering compounds coming from the matrix which are not removed by the cleanup procedure.

Table 2 Experimental conditions for the electron ionization–multiple reaction monitoring method

Compound	Retention time (min)	Precursor ion (*m*/*z*)[b]	CE (eV)	Product ion (*m*/*z*)	*Q*/*q*[c]
α-BHC	7.5	181	20	145	2.0 (4)
		219	20	147	
-BHC	8.1	181	20	145	1.7 (3)
		218	20	147	
β-BHC	8.8	181	20	145	1.7 (6)
		219	20	147	
δ-BHC	9.4	181	20	145	1.9 (5)
		219	20	147	
Heptachlor	10.2	100	20	65	1.5 (1)
		272	20	237	
Aldrin	11.2	263	30	193	1.2 (10)
		293	30	222	
Chlorpyrifos	11.5	314	25	258	1.7 (1)
		197	25	169	
Heptachlor epoxide	12.7	353	30	263	2.1 (4)
		353	20	219	
Chlorfenvinfos-D_{10}[a]	12.9	323	25	268	1.3 (9)
Chlorfenvinfos	13.1	323	25	267	1.6 (4)
		323	25	159	
-Chlordane	13.6	373	30	266	1.5 (12)
		373	30	264	
α-Endosulfan	14.2	241	25	206	1.3 (5)
		207	20	172	
α-Chlordane	14.3	373	30	266	1.3 (5)
		373	30	264	
BPA-D_{16}[a]	15.3	224	20	125	1.1 (7)
BPA	15.4	213	15	119	1.8 (8)
		213	15	91	
DDE-D_{8a}	15.4	254	20	184	1.4 (11)
Dieldrin	15.5	79	15	77	1.9 (6)
		79	15	51	
DDE	15.6	318	20	246	1.3 (7)
		318	20	283	
Endrin	16.0	263	20	193	1.3 (3)
		263	20	228	
β-Endosulfan	16.3	195	15	125	2.4 (5)
		237	15	143	
DDD	16.6	235	20	165	1.2 (7)
		235	20	199	
Endrin aldehyde	16.8	250	10	215	2.3 (5)
		345	10	245	
Endosulfan sulfate	17.3	272	20	237	1.4 (2)
		387	20	241	
DDT-$\boldsymbol{D_8}$[a]	17.4	243	20	173	2.0 (3)
DDT	17.5	235	20	165	2.7 (4)
		235	20	199	
Endrin ketone	18.1	317	10	101	1.7 (2)
		317	10	245	
Methoxychlor-$\boldsymbol{D_{14}}$[a]	18.3	241	25	177	1.9 (5)
Methoxychlor	18.4	227	25	168	1.5 (2)
		227	25	212	
Boscalid	20.6	140	15	76	1.2 (3)
		140	15	112	

BPA bisphenol A, *CE* collision energy

[a] Isotopically labeled standard

[b] The first line gives the quantification transition (Q), and the second line gives the confirmation transition (q).

[c] Average value of three injections of standard solutions (three replicates each) and the relative standard deviation in *parentheses*

Method validation

Validation of the method considered the following parameters: LOD, limit of quantification (LOQ), linearity (R^2), intraday repeatability, interday repeatability, trueness (recovery), and precision. The LODs were defined as a signal-to-noise ratio of 3 or more for the less intense (confirmation) MRM transition. They ranged from 0.03 to 0.1 ng g^{-1} for each compound (Table 3). The LOQs (signal-to-noise ratio of 10 or more) ranged from 0.2 to 0.6 ng g^{-1}(Table 3). Thus, the analytical method proved to be sensitive enough.

Linearity

The method linearity was evaluated using samples (0.5 g) of lamb cerebral cortex. All samples were previously tested to assess the absence of the selected compounds. This procedure was applied to each brain sample batch used to validate the method. Samples of blank tissues were fortified at seven concentrations ranging between 0.2 and 200 ng g^{-1}, as established in Commission Decision 2002/657/EC [51]. The first four levels of calibration (0.2, 2.0, 10.0, 20.0 ng g^{-1}) were chosen to give more importance to the low concentrations, as suggested by Antignac et al. [52]. Each concentration was injected in triplicate. Internal standard linear calibration curves were plotted by least-squares regression of concentration versus the relative peak area (analyte/internal standard) of the calibration standards. Adequate linearity values, calculated as a determination coefficient (R^2) higher than 0.9940, were obtained for all compounds (Table 3).

Trueness and precision

Trueness (recovery) and precision were evaluated by means of recovery experiments (n=9) at three concentrations (1, 10, 50 ng g^{-1}). This range of concentrations was chosen since it was close to the expected concentrations in real samples. Each lamb matrix was spiked and extracted three times. As can be observed in Fig. 1, the histogram shows that most compounds had satisfactory recoveries, with values ranging between 70 and 120 % at the three fortification levels. Precision was expressed as the intraday and interday repeatability (relative standard deviation). The intraday repeatability was estimated at each fortification level from the recovery experiments (n=

Table 3 Limit of detection (*LOD*), limit of quantification (*LOQ*), and calibration parameters in lamb cerebral cortex

Compound	Internal standard	LOD (ng g^{-1})	LOQ (ng g^{-1})	Equation	R^2
α-BHC	DDE-D_8	0.07	0.21	y=7.0x+0.01	0.9956
-BHC	DDE-D_8	0.07	0.25	y=6.04x+0.001	0.9946
β-BHC	DDE-D_8	0.03	0.13	y=3.2x−0.01	0.9942
δ-BHC	DDE-D_8	0.07	0.20	y=4.1x−0.002	0.9970
Heptachlor	DDE-D_8	0.07	0.21	y=4.05x+0.003	0.9960
Aldrin	DDE-D_8	0.07	0.22	y=1.05x−0.002	0.9957
Chlorpyrifos	Chlorfenvinfos-D_{10}	0.03	0.17	y=2.1x−0.01	0.9988
Heptachlor Epoxide	DDE-D_8	0.10	0.32	y=0.06x+(2.4×10^4)	0.9944
Chlorfenvinfos	Chlorfenvinfos-D_{10}	0.07	0.24	y=1.3x+0.006	0.9945
-Chlordane	DDT-D_8	0.03	0.13	y=0.25x+(4.5×10^4)	0.9945
α-Endosulfan	DDT-D_8	0.20	0.61	y=0.38x+(1.2×10^4)	0.9951
α-Chlordane	DDT-D_8	0.07	0.20	y=0.14x+(4.5×10^4)	0.9945
BPA	BPA-D_{16}	0.07	0.21	y=4.4x+0.05	0.9754
Dieldrin	DDT-D_8	0.10	0.33	y=0.40x+0.01	0.9936
DDE	DDE-D_8	0.07	0.22	y=2.70x−(6.4×10^4)	0.9949
Endrin	DDT-D_8	0.07	0.24	y=0.06x+(1.9×10^4)	0.9928
β-Endosulfan	DDT-D_8	0.07	0.23	y=0.03x+(1.9×10^4)	0.9948
DDD	DDT-D_8	0.07	0.27	y=1.80x+0.005	0.9944
Endrin aldehyde	Methoxychlor-D_{14}	0.07	0.24	y=0.02x+(1.4×10^4)	0.9949
Endosulfan sulfate	Methoxychlor-D_{14}	0.10	0.31	y=0.11x−(6.5×10^4)	0.9891
DDT	DDT-D_8	0.07	0.26	y=0.47x+0.002	0.9962
Endrin ketone	Methoxychlor-D_{14}	0.07	0.21	y=0.08x+(3.6×10^4)	0.9955
Methoxychlor	Methoxychlor-D_{14}	0.03	0.17	y=1.45x−(9.4×10^4)	0.9943
Boscalid	Chlorfenvinfos-D_{10}	0.03	0.17	y=7.009431x+0.013391	0.9956

BPA bisphenol A

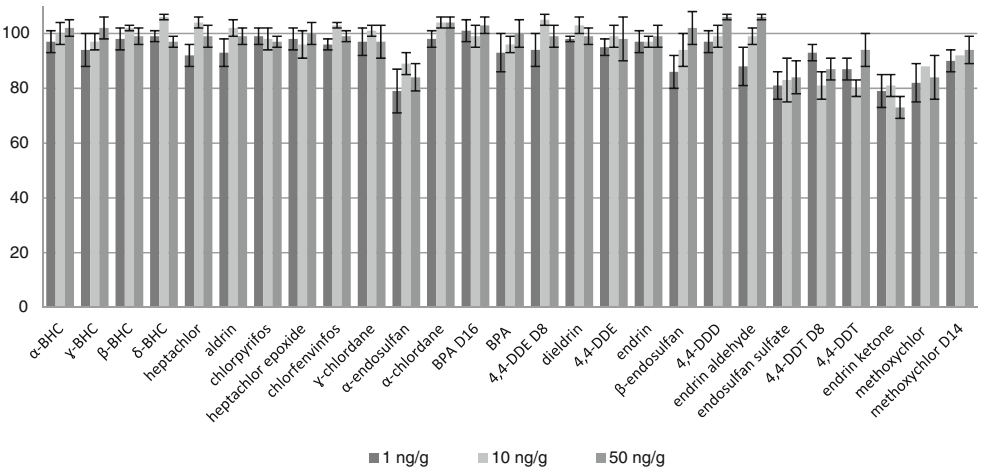

Fig. 1 Histogram obtained from the recovery experiments for the matrices fortified at 1, 10, and 50 ng g^{-1}. *BPA* bisphenol A

3). A 50 pg μL^{-1} standard solution was prepared daily for the interday repeatability studies, and five injections were made for each day (5 days, n=25). The intraday repeatability ranged from 2 to 6 % for all injected concentrations and the interday repeatability ranged from 8 to 18 %. The results obtained indicate the good repeatability and accuracy of the proposed method.

Application to real samples

Highly selective detectors such as MS/MS analyzers are recommended in the analysis of complex matrices. They allow one to discriminate between the interferences coming from the matrix (i.e., biological tissues) which are not eliminated by the cleanup procedure and to identify and quantify analytes even at very low concentrations. The use of MRM mode permits the monitoring of specific transitions (specific precursor and product ions), and is able to unequivocally detect all compounds of interest. The method was applied to the analysis of 14 human cerebral cortexes coming from ten SIUDS and four SIDS victims. The presence of some environmental contaminants detected in eight positive samples from 14 fetal and neonatal brain tissues, as reported in Table 4, could lead to the possibility that they can pass from mother to fetus and can be collected in the brain tissue. This is in accordance with literature data that report the presence of the selected pollutants in other human tissues [53–55]. It is very interesting to note that four brain samples showed the presence at parts per billion levels of several OCPs and three samples evidenced the presence of two OPPs—chlorpyrifos and chlorfenvinfos—the commonest pesticides used for many crops, such as apples and grapes, in several Italian regions. Even though these compounds are environmentally nonpersistent, their extensive use in pest control exposes the population to continuous low-level concentrations. The very low concentrations of some contaminants found in a few samples can be of great concern owing to the delicacy of fetal and newborn tissues. As a reference, for an adult, the acceptable daily intake of these compounds ranges from 0.002 to 0.02 mg per kilogram of body weight.

As regards the confirmation of positive findings, all the compounds with a high concentration (above the LOQ) as well as those detected at low concentration (between the LOD and the LOQ) were confirmed by the use of the two transitions selected, the compliance of Q/q ratios, and retention times. As an example, Fig. 2 shows the MS/MS chromatograms, with relative experimental Q/q ratios, for two of the compounds detected in two brain samples from SIDS (Fig. 2a, case N12) and SIUDS (Fig. 2b, case F11) victims, respectively. The Q/q ratios calculated for all positive samples were within the range of the tolerance accepted around the experimental average Q/q value obtained from reference standards. The application of this method allowed the quantification and confirmation of all selected compounds at the 0.2–0.6 ng g^{-1} level. The use of MS/MS in QqQ instruments working in MRM mode permits the rapid, efficient, and sensitive confirmation of these environmental contaminants.

Conclusions

A GC–MS/MS method for quantification and confirmation of selected environmental contaminants in human fetal and newborn brain tissues has been proposed. The use of a QqQ analyzer, working in MRM mode, allowed us to achieve excellent selectivity and sensitivity compared with our previous work. The selectivity was improved by recording two transitions, with two different precursor ions, for most of the analytes. The ratios obtained from them were used as confirmatory parameters, together with the retention time. The application of this method to a real matrix allowed the quantification and confirmation of all selected compounds at the 0.2–0.6 ng/g level. Finally, the method was applied to 14 real samples from ten SIUDS and four SIDS victims. Even though the selected contaminants were detected only at low parts per billion

Table 4 Target compounds in brain tissues from SIUDS and SIDS victims

Sample label	Description	Compound	Concentration(ng/g)	Experimental *Q*/*q*	Theoretical *Q*/*q*
F3	SIUDS (35 weeks), female	Methoxychlor	0.2	1.55	1.52
F4	SIUDS (34 weeks and 5 days), female	Methoxychlor	<LOQ[a]	1.57	1.52
F6	SIUDS (40 weeks and 4 days), male	DDE	<LOQ[a]	1.36	1.32
F8	SIUDS (49 weeks), male	Chlorpyrifos	0.4	1.68	1.72
F9	SIUDS (39 weeks and 3 days), female	β-Endosulfan	2.8	2.23	2.45
F11	SIUDS (34 weeks and 1 day), female	Chlorfenvinfos	9.9	1.67	1.60
N12	SIDS (4 months), female	DDE	0.6	1.31	1.32
		Chlorpyrifos	2.1	1.75	1.72
		Heptachlor	NC[b]	–	
		DDD	NC[b]	–	
		Methoxychlor	NC[b]	–	
		γ-BHC	NC[b]	–	
N14	SIDS (4 months), male	Heptachlor	NC[b]	–	
		Chlorfenvinfos	0.5	1.53	1.60
		α-Chlordane	NC[b]	–	
		DDE	0.4	1.39	1.32
		β-Endosulfan	3.1	2.51	2.45

NC not confirmed owing to the low response of the confirmatory transition (q)

[a] Detected but not quantified

[b] Between the LOD and the LOQ

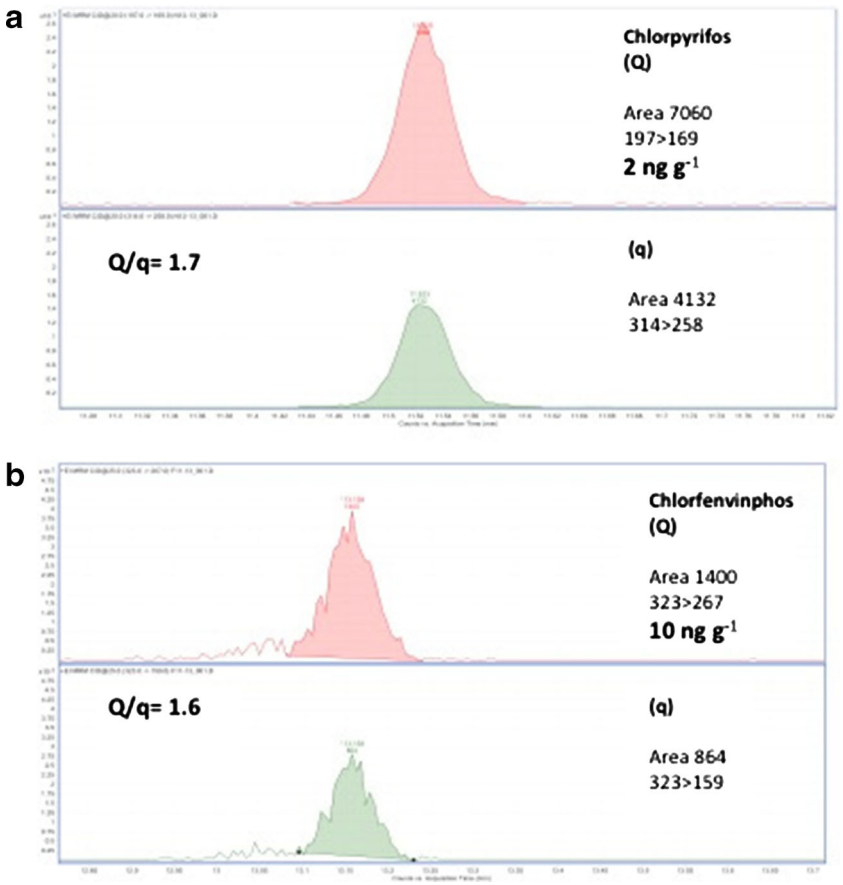

Fig. 2 Multiple reaction monitoring chromatograms for two compounds detected in two brain samples from sudden infant death syndrome (**a**, case N12) and sudden intrauterine unexpected death syndrome (**b**, case F11) victims. *Q* quantification transition, *q* confirmation transition

levels, the assessment of possible fetal and newborn exposures, together with the determination of these compounds in fetal and newborn tissues, is a critical factor to establish a potential correlation with sudden death syndromes.

The method will allow future developments in establishing a possible correlation between environmental contaminants and the syndromes. In fact, it is a robust platform for the analysis of a large number of samples and offers the possibility to extend the study to other classes of pollutants such as polychlorinated biphenyls and phthalates.

Acknowledgments The authors thank Agilent Technologies for providing the GC–MS/MS instrument. We are very grateful to all the personnel in the pathology units of Rovereto, Lecco, and Modena hospitals for providing SIUDS and SIDS autopsy samples.

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