Environmental and industrial toxicology

QUANTITATIVE DETERMINATION OF EIGHT ORGANOCHLORINE PESTICIDES IN SERUM BY GC COUPLED TO TANDEM MASS SPECTROMETRY

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Key words: Organochlorine pesticides, GC-MS/MS method, validation, uncertainty estimation, total error

ABSTRACT

The pathogenic role of exposure to organochlorine pesticides residues is a matter of controversy. Some of them were recently suspected to be associated with neurodegenerative diseases (Parkinson, Alzheimer, ...), especially α-hexachlorocyclohexane (α-HCH), β-HCH, γ-HCH, p,p'-1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE), o,p'-DDE, dieldrin, p,p'-dichlorodiphenyltrichloroethane (p,p'-DDT) and o,p'-DDT. Since these organochlorine products are prohibited in Europe and USA, the blood concentrations found in general population are usually lower than 1µg/L for each compound. To allow quantification of very low concentrations, the analytical method needs to be as sensitive as possible. A gas chromatography coupled to tandem mass spectrometry method has been developed for the simultaneous determination of the eight residues, and has been validated in order to check its suitability with our objectives.

Sample preparation included a double liquid-liquid extraction of the serum with a mixture of petroleum ether and diethyl ether followed by a solid phase extraction on Bond Elut LRC-Certify cartridges (130mg, Varian). Extracted sample was injected onto the gas chromatography analyzer (Agilent, 7890A). The chromatographic separation was done on a HP-5MS column (30m x 0.25mm, 0.25µm, Agilent) and compounds were then analyzed in the tandem mass spectrometer (Agilent, Triple Quad, 7000A). Two transitions were studied by molecule. The method was validated using total error approach.

The linearity of the method was acceptable in the validated range of concentrations for the eight pesticides. The bias was lower than 10%, the relative standard deviations are lower than 10% for repeatability (n=3) and than 15% for intermediate precision (k=3). Lower and upper β-expectation tolerance limits did not exceed the acceptance limits of 20%. The limit of quantitation was about 0.5µg/L for all compounds.

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The gas chromatography coupled to tandem mass spectrometry (GC-MSMS) technology was fully convenient for the detection and the quantitative determination of 8 organochlorine residues in serum. The method had been applied to the identification and quantification of the products in blood samples obtained from patients suffering from neurodegenerative disorders.

INTRODUCTION

Exposure to organochlorine pesticides residues has been suspected since many years to increase the incidence of several endocrine pathologies (1,2,3,4,5). Organochlorine pesticides have also been demonstrated to be neurotoxic (6,7,8,9), to cause oxidative stress and to damage the dopaminergic system in the rodent brain. The combination of their persistence in the environment and their potential to damage the dopamine system suggested that organochlorine pesticides may be significant contributors to the observed association between pesticide exposure and risk of Parkinson disease (6).

Dieldrin, dichlorodiphenyltrichloroethane (DDT) and lindane were used as insecticides around the world until the middle 1970s. They are forbidden in the USA and Central Europe since 1980s but because of their long remanence time, general population is still exposed to these residues (1). DDT exists in isomeric forms, p,p'-DDT and o,p'-DDT which are respectively transformed by the liver into p,p'-1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE) and o,p'-DDE. (1). β-hexachlorocyclohexane (β-HCH) is a persistent ingredient in technical-grade lindane (γ-HCH) but both isomers of hexachlorocyclohexane (HCH) have been demonstrated to cause oxidative stress and to reduce brain concentrations of dopamine in animals (6). Richardson et al. associated exposure to pesticides with increased risk of Parkinson disease and concluded that elevated levels of β-HCH were linked with the diagnosis of Parkinson disease. They also found significantly higher levels of p,p'-DDE in patients serum with Alzheimer disease versus controls (6).

Kanthasamy et al. suggested that dieldrin induced apoptic cell death, altered dopamine levels, mitochondrial dysfunction and protein aggregation; therefore, dieldrin possessed key properties that characterized it as a dopaminergic toxin (9).

In order to establish contamination level in a population of people suffering from neurodegenerative disorders, we developed the quantitative determination in serum of eight organochlorine pesticides for which neurotoxicity has been suspected: α-HCH, β-HCH, γ-HCH, dieldrin, o,p'-DDT, p,p'-DDT, o,p'-DDE and p,p'-DDE. The method validated with a triple quadruprole GC-MS system was satisfying in terms of performances and sensitivity.

MATERIALS AND METHODS

Chemicals and reagents

Pesticides reference standards for all compounds and internal standards were purchased from Dr Ehrenstorfer (Augsburg, Germany). All standards had a purity upper than 98%.

Petroleum ether, diethyl ether and n-hexane were purchased from J.T. Baker (Deventer, the Netherlands), acetonitrile and methanol were from Biosolve (Valkenswaard, the Netherlands), potassium carbonate and sodium sulphate were from Sigma (Steinheim, Germany). All reagents were at least of analytical grade. Solid phase extraction (SPE) cartridges, Bond Elut LRC-Certify 130mg, were obtained from Varian Chrompack (Palo Alto, CA).

Stock solutions and standards

A mixture of aldrin, α-HCH-d_6, γ-HCH-d_6, p,p'-DDE-d_8, p,p'-DDT-d_8 prepared in hexane at the concentration of 100µg/L was used as internal standard.

Calibration standards and validation standards were prepared by spiking serum with stock solution (100µg/L) containing the 8 organochlorine pesticides. Calibration standards were prepared at the concentrations of 1, 2, 4 and 8µg/L; they were analyzed in duplicate for three days and were used to establish the best response function. Validation standards were prepared at the concentrations of 0.5, 1, 2, 5 and 10µg/L, they were analyzed in triplicate for three days and were used to estimate the method limits. An extract of blank serum was also prepared for each run.

Sample preparation

Twenty-five µL of internal standard solution were added to 500µL of serum. Five hundred µL of acetonitrile were added in order to precipitate proteins followed by 500µL of saturated potassium carbonate ; this mixture was extracted two times with 5mL of a mixture of petroleum ether and diethyl ether (v/v : 98/2). Organic phase was recovered and purified by solid phase extraction. SPE cartridges (Bond Elut LRC-

Certify, 130mg, Varian) were conditioned with 3mL of methanol, Na_2SO_4 and 3mL of the petroleum ether/diethyl ether mixture. After purification, the organic phase was evaporated to dryness under gentle nitrogen flow at 40°C and reconstituted with 50µL of hexane (10), 2 µl were injected in pulsed splitless mode (30psi) onto the GC.

Instrumentation

Analysis was performed on a gas chromatography analyzer GC 7890A coupled to a mass spectrometer MS Triple Quad 7000A equipped with a multimode injector (Agilent , Waldbronn, Germany). The chromatographic separation was done on a HP-5MS column (30m x 0.25mm i.d., 0.25µm film thickness, Agilent) using the following temperature program: initial temperature 55°C held for 1.5 min., increased to 150°C at a rate of 60°C/min, then increased to 200°C at a rate of 6°C/min, then increased to 280°C at a rate of 280°C, and held for 1.5 min. The carrier gas was helium maintained at a constant flow of 1.2 mL/min.

After chromatographic separation, compounds were analyzed in the tandem mass spectrometer operated in the electronic impact mode (70eV) in the MS/MS mode. Two multiple reaction monitoring (MRM) were studied by molecule for identification and quantification (see Table1).

Method validation

According to ISO17025 and the guidelines of the French Society of Pharmaceutical Sciences and Techniques (SFSTP), the present method was fully validated using total error approach (11,12,13). The e-noval software V2.0 (Arlenda, Liège, Belgium) was used to compute all validation results and to build the accuracy profiles.

RESULTS

Chromatographic time was 18 minutes. The eight compounds were well separated, with retention times from 11 to 17.5 min. A chromatogram of an extracted spiked serum (5µg/L of each pesticide) is presented in Figure1.

The response function is, within the range, the existing relationship between the response (signal) and the concentration of the analyte in the sample. (12). It was build from the calibration standards. The response function was a linear regression for β-HCH, dieldrin and o,p'-DDT, a weighted (1/x) quadratic regression for α-HCH, γ-HCH and p,p'-DDT, a weighted linear regression for o,p'-DDE and p,p'-DDE.

Table 1 – MRM and internal standard for 8 organochlorine pesticide residues

Compound	Internal std	MRM1	Collision energy (V)	MRM2	Collision energy (V)
α-HCH-d_6	-	223.9>187.0	5	184.9>150.0	15
α-HCH	α-HCH-d_6	218.8>183.0	5	180.9>145.0	12
β-HCH	α-HCH-d_6	218.8>183.0	5	180.9>145.0	12
γ-HCH-d_6	-	223.9>187.0	5	184.9>150.0	15
γ-HCH	γ-HCH-d_6	218.8>183.0	5	184.9>150.0	12
Aldrin	-	262.8>193.0	35	262.8>191.0	30
Dieldrin	Aldrin	262.8>193.0	35	262.8>191.0	35
o,p'-DDE	p,p'-DDE-d_8	247.9>176.0	30	245.9>176.0	30
p,p'-DDE-d_8	-	255.9>184.0	33	253.9>184.0	33
p,p'-DDE	p,p'-DDE-d_8	247.9>176.0	30	245.9>176.0	30
o,p'-DDT	p,p'-DDT-d_8	236.9>165.0	25	234.9>165.0	25
p,p'-DDT-d_8	-	244.9>173.2	25	242.9>173.2	25
p,p'-DDT	p,p'-DDT-d_8	236.9>165.0	25	234.9>165.0	25

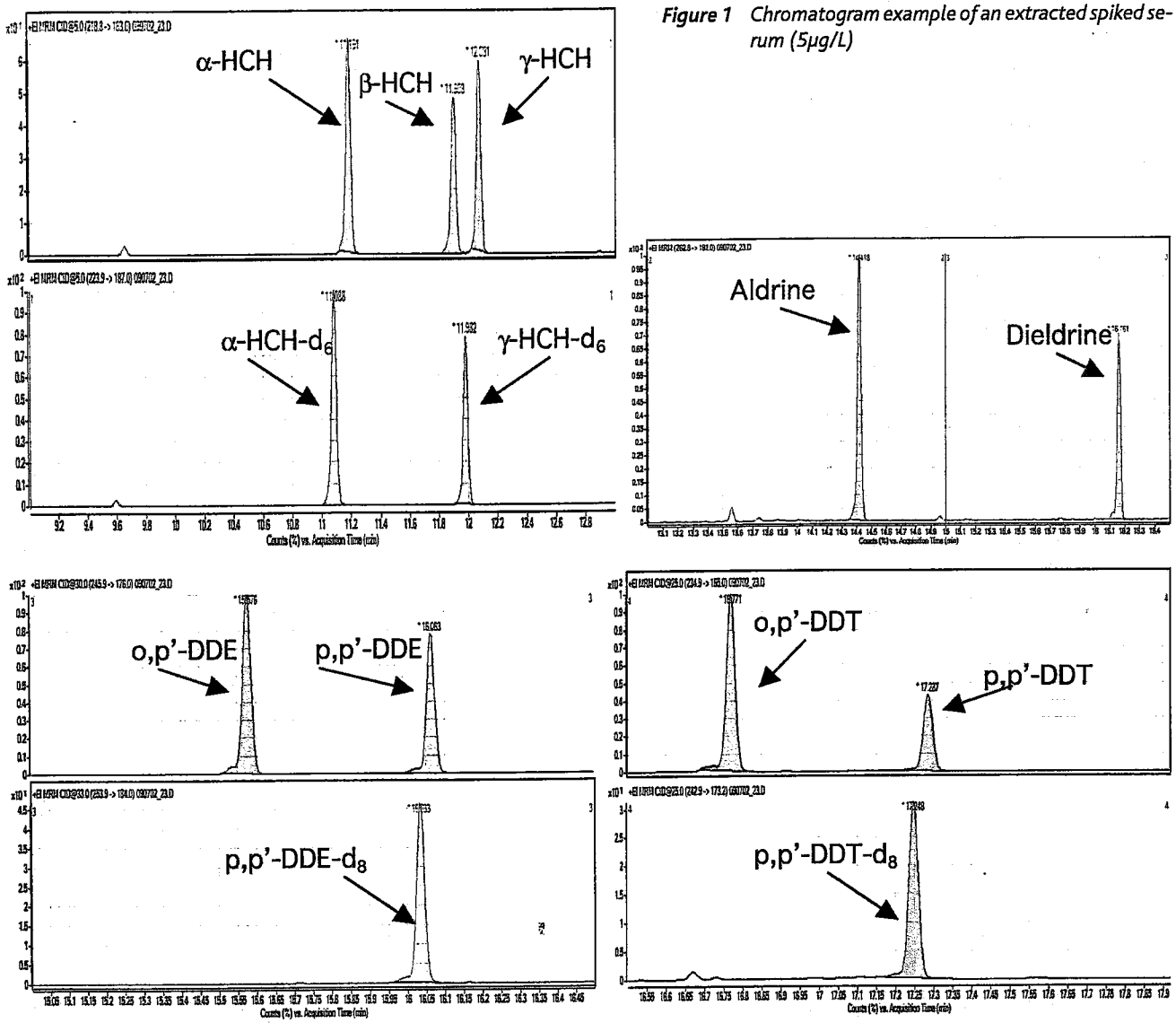

Figure 1 *Chromatogram example of an extracted spiked serum (5µg/L)*

The linearity is the method ability to obtain results directly proportional to the concentrations of the analyte in the sample (12). The method presented a good linearity in the validated range for each compound.

The trueness expresses the closeness of agreement between the mean value obtained from the validation standards and the value which is accepted either as a conventional true value or an accepted reference value. Trueness is expressed in terms of relative bias (systematic error) (12). Trueness was acceptable for all organochlorine residues, since the relative bias were always smaller than 8%. Results are presented in Table2.

The precision was determined by computing the Relative Standard Deviations (RSDs) for repeatability and intermediate precision at each concentration level of the validation standards (14). They did not exceed 7% for repeatability and 11% for intermediate precision. RSDs are presented in Table2.

The uncertainty characterizes the dispersion of the values that could reasonably be attributed to the measurand. The expanded uncertainty represents an interval around the results where the unknown true value can be observed with a confidence level of 95%. The relative expanded uncertainties (%) are obtained by dividing the corresponding expanded uncertainties with the corresponding introduced concentrations. Values for each pesticide are presented in Table2.

Ability of the method to produce accurate results was evaluated by total error calculation. Thus, the total error estimation of a procedure is fundamental to assess the validity of the method. Total error is the sum of trueness and precision, and is clearly a good indi-

Table 2 – Trueness, precision, uncertainty for 8 organochlorine pesticide residues

	Target conc. µg/L	α-HCH	β-HCH	γ-HCH	o,p'-DDE	p,p'-DDE	Dieldrin	o,p'-DDT	p,p'-DDT
Trueness Relative bias (%)	0.5	7.10	2.51	6.73	4.78	3.32	7.62	-1.95	3.83
	1.0	-1.45	-5.97	-3.38	-0.05	-0.44	-3.09	-6.20	-1.52
	2.0	0.82	-7.33	-2.04	2.51	0.14	-5.47	-4.36	-2.12
	5.0	0.94	-3.73	0.89	2.69	0.26	-5.57	-0.92	2.04
	10.0	0.16	-0.16	0.62	3.59	1.00	-2.29	2.44	1.69
Intra-assay precision Repeatability (RSD%)	0.5	4.95	5.80	2.16	4.60	5.43	6.67	4.00	6.83
	1.0	0.77	2.54	1.07	3.19	2.14	3.32	1.16	1.75
	2.0	0.95	2.99	1.56	2.34	1.85	3.70	3.44	4.01
	5.0	1.70	3.36	0.78	1.50	1.59	1.76	4.29	3.28
	10.0	2.16	2.62	1.19	1.39	1.32	2.29	3.25	4.09
Inter-assay precision Intermediate precision (RSD%)	0.5	4.95	9.33	3.07	5.85	6.05	6.83	10.55	6.83
	1.0	1.90	3.20	2.56	3.19	2.40	3.45	1.21	3.62
	2.0	2.58	2.99	1.56	2.59	1.85	5.16	3.46	4.01
	5.0	4.60	4.91	3.59	2.40	2.01	4.73	7.28	5.98
	10.0	3.97	4.69	4.05	1.39	1.41	3.00	3.90	4.18
Uncertainty Relative expended uncertainty (%)	0.5	10.44	20.85	6.79	12.79	12.99	14.47	24.08	14.40
	1.0	4.32	6.98	5.84	6.73	5.17	7.33	2.57	8.19
	2.0	5.88	6.30	3.28	5.55	3.90	11.39	7.32	8.45
	5.0	10.49	10.89	8.58	5.35	4.39	10.79	16.39	13.45
	10.0	8.94	10.55	9.28	2.92	3.02	6.58	8.56	8.85

cator of results accuracy. The accuracy expresses the closeness of agreement between the value found and the value which is accepted either as a conventional true value or an accepted reference value (13,14).

The accuracy profile is obtained by joining the extremes of the 90% interval, i.e. the interval that will contain 90% of the future individual results. The acceptance limits were set at ± 20%. As shown in Figure 2, the relative upper and lower β-expectation tolerance intervals did not exceed the acceptance limits for each compound in the dosing range.

The intersection between the accuracy profile and the acceptance limits defines the lower limit of quantitation (LQL) as well as the upper limit of quantitation (UQL) (13,14). LQL and UQL of the 8 organochlorine pesticides are presented in Table3.

DISCUSSION

Sample preparation consisted in a double liquid-liquid extraction followed by a solid-phase extraction. Chromatographic time was 18 minutes. The MS method was divided into 4 separate periods of time; from 9 to 13 minutes for α-HCH, α-HCH-d_6, β-HCH, γ-HCH and γ-HCH-d_6; from 13 to 15 minutes for aldrin; from 15 to 16.5 min for dieldrin, o,p'-DDE, p,p'-DDE-d_8 and p,p'-DDE and finally from 16.5 minutes to the end of the chromatography for o,p'-DDT, p,p'-DDT-d_8 and p,p'-DDT.

The quantitative analysis of 8 organochlorine pesticides answered to our objectives. Response functions were a linear regression or a quadratic regression, weighted or not, depending on the analyte. Linearity was respected from LOQ to 10µg/L for each compound. Bias ranged from -7.3% to 7.1% for α-HCH, β-HCH and γ-HCH, from -0.4% to 4.9% for o,p'-DDE and p,p'-DDE, from -6.2% to 3.8% for o,p'-DDT and p,p'-DDT and from -5.6% to 7.6% for dieldrin. For repeatability, RSDs were lower than 5 % for o,p'-DDT and p,p'-DDT, lower than 6% for α-HCH, β-HCH, γ-HCH, o,p'-DDE and p,p'-DDE and lower than 7% for dieldrin. For reproducibility, RSDs were lower than 7% for o,p'-DDE, p,p'-DDT and dieldrin and were of approximately 10% for α-HCH, β-HCH, γ-HCH, o,p'-DDT and p,p'-DDT. The lower and upper β-expectation tolerance limit did not exceed the acceptance limits (20%) at 90% level and the lower limit of quantitation allowed measurement of pesticides concentrations in serum of healthy individuals, generally lower than 1µg/L.

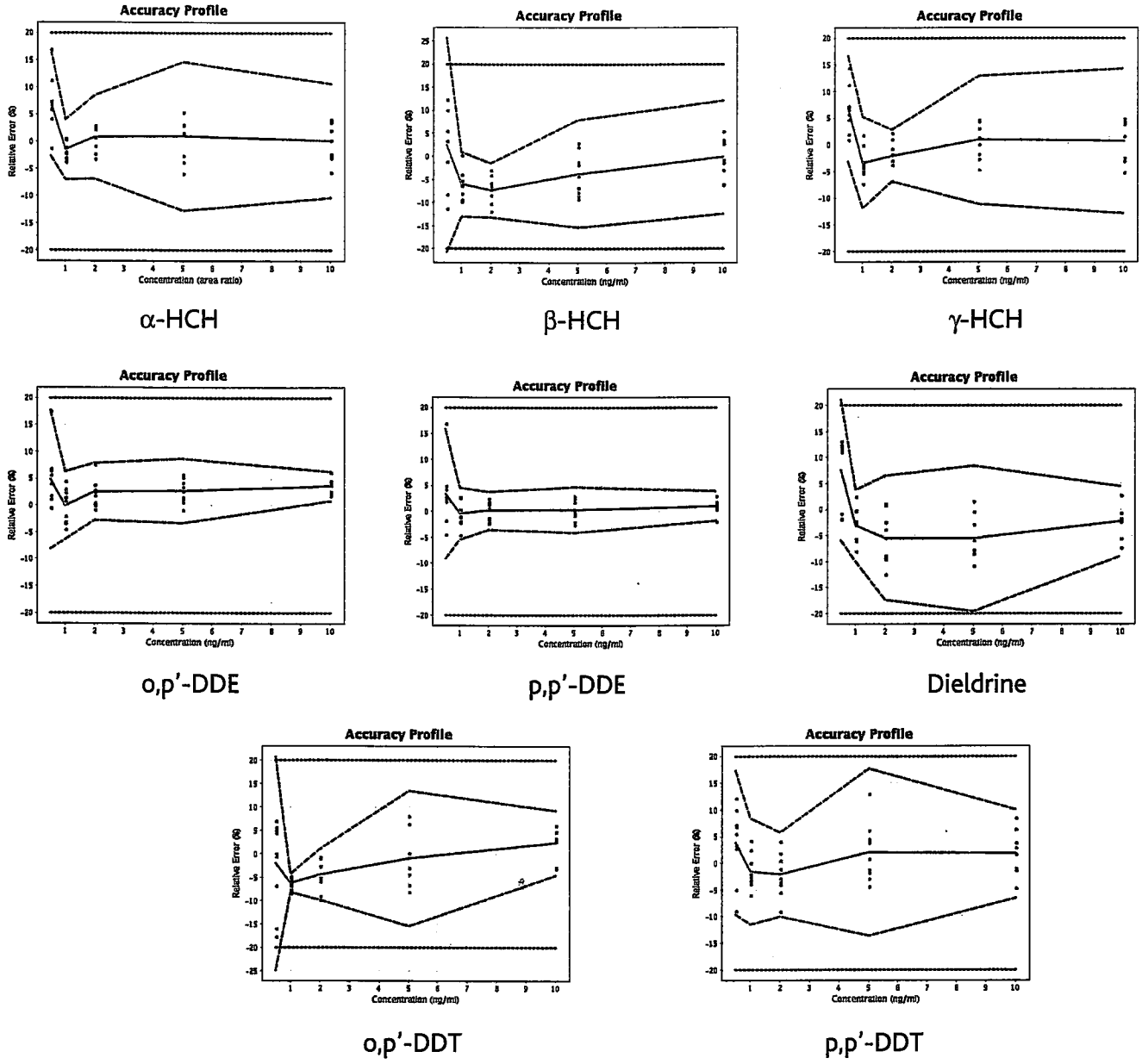

Figure 2 *Accuracy profiles for 8 organochlorine pesticide residues*
Legend: The plain line is the relative bias, the dashed lines are the b-expectation tolerance limits and the dotted curves represent the acceptance limits (20%). The dots represent the relative back-calculated concentrations and are plotted with respect to their targeted concentration.

Table 3 – Lower and upper limits of quantitation for 8 organochlorine pesticide residues

Compound	LQL (µg/L)	UQL (µg/L)
α-HCH	0.5	10.0
β-HCH	0.6	10.0
γ-HCH	0.5	10.0
o,p'-DDE	0.5	10.0
p,p'-DDE	0.5	10.0
Dieldrin	0.5	10.0
o,p'-DDT	0.6	10.0
p,p'-DDT	0.5	10.0

CONCLUSION

To allow quantification in human blood of 8 organochlorine pesticides suspected to be neurotoxic, we developed a GC-MS/MS method performed after a double extraction. The method was fully validated using total error approach which is a really innovative procedure for analytical validation in toxicological laboratories. Gas chromatography coupled to tandem mass spectrometry is very selective and sensitive and offers many new possibilities compared to previously

used technologies (ion trap, ECD). Our method showed limits of quantitation of ± 0.5µg/L, which is better than the previously published methods (10). Other Persistent Organic Pollutants (POPs) will be added to the method in a near future.

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