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Simultaneous analysis of chlorpyrifos and cypermethrin in cord blood plasma by online solid-phase extraction coupled with liquid chromatography–heated electrospray ionization tandem mass spectrometry

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ABSTRACT

Chlorpyrifos and cypermethrin are the most used insecticides in Taiwan. Exposure to both pesticides has been associated with reproductive and developmental health effects in humans and animals. This study describes an online solid-phase extraction coupled with liquid chromatography–heated electrospray ionization tandem mass spectrometry (online SPE-LC/HESI/MS/MS) method to analyze chlorpyrifos and cypermethrin in cord blood of pregnant women. Calibration curves showed good linearity ($r^2 > 0.998$) for both pesticides within the range of 0.1–100 ppb. Limits of detection (LODs) were 0.01 and 0.05 ppb and recoveries in cord blood were 97.2 ± 4.8% and 93.5 ± 9.5% for chlorpyrifos and cypermethrin respectively. After analysis of 396 samples, the mean concentrations of chlorpyrifos and cypermethrin were 0.38 and 1.08 ppb respectively. These results demonstrate that LC/HESI/MS/MS is effective for the simultaneous analysis of chlorpyrifos and cypermethrin in cord blood with excellent sensitivity and specificity and may also be effective for high throughput assay in future epidemiology studies.

1. Introduction

Pesticides are widely used in agriculture and environmental health to prevent or destroy pests on crops and in houses all over the world. In Taiwan, chlorpyrifos and cypermethrin are the most frequently used insecticides in agriculture and in households [1]. Residues have been detected in a range of samples including crops, fish, water, soil, and the air in residential houses, schools, and parks. People may be exposed to the compounds through different routes of dietary intake, environmental and occupational contact [2,3].

Chlorpyrifos in maternal and cord plasma has been associated with adverse birth outcome and deficits in neurodevelopment [4–8]. Cypermethrin has also been reported to be more toxic to neonates than adult rats due to lack of specific enzymes which catalyze the ester hydrolysis of pyrethroids [9–11]. The potential health risk resulting from exposures to chlorpyrifos and cypermethrin is therefore of significant concern to pregnant woman and the developing child.

In order to assess the total exposure to these pesticides from different routes, analysis of their parent compounds or corresponding metabolites in blood or body fluid has been considered an appropriate approach [12–14]. Recently, liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) has been applied to develop analytical methods to monitor pesticides as indicators of physiological exposure [14]. LC/MS/MS provides capacity for simultaneous measurement of multiple analytes with excellent specificity and sensitivity.

Although gas chromatography–mass spectrometry (GC/MS) still remains the most popular method for analysis of cypermethrin [19,20], LC/MS/MS offers some advantages over GC/MS, especially in simplified sample preparation. HPLC methods have also been developed to analyze cypermethrin in blood with limits of detection (LODs) higher than 1 ppb [17,18]. After precipitation of proteins in blood samples, chlorpyrifos has been quantitated with LC/MS/MS directly or following column liquid chromatography. LODs of these methods ranged from sub-ppb to 1.5 ppb with an analysis time of approximately 8 min [15,16].

To simultaneously analyze the extremely low concentrations of chlorpyrifos and cypermethrin in cord blood, a method with excellent sensitivity and specificity is needed. Such a method must also be conserving of the small volume of sample available and therefore include an effective sample preparation step. Sample preparation procedures, such as liquid–liquid extraction (LLE), solid-phase extraction (SPE), and solid-phase microextraction (SPME), would be required to remove the proteins and interfering components

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in blood samples, but are usually sample-spending and time-consuming.

Therefore, the objective of this study was to develop an online SPE coupled with liquid chromatography–heated electrospray ionization tandem mass spectrometry (online SPE-LC/HESI/MS/MS) method to simultaneously analyze chlorpyrifos and cypermethrin in cord blood. By using this method, chlorpyrifos and cypermethrin can be analyzed effectively and rapidly may potentially serve as a high throughput assay for future epidemiology studies on the potential health effects caused by the exposure to chlorpyrifos and cypermethrin.

2. Materials and methods

2.1. Chemicals

Chlorpyrifos was purchased from Chem Service (West Chester, PA, USA). Cypermethrin was obtained from Riedel-de Haën (Seelze, Germany). Methanol was purchased from J.T. Baker (Philipsburg, NJ, USA). Ammonium acetate was bought from Fluka (Buchs, SG, Switzerland).

2.2. Method validation

Individual stock solutions were prepared by dissolving 10 mg of each pesticide into 10 ml pure methanol and stored at −20 °C until used. The working standards were prepared by serial dilution of stock solutions with methanol and ranged from 0.1 to 100 ppb. After analysis of these working standard solutions, calibration curves were then established by plotting the peak areas versus the concentrations of these standards. The limit of detection (LOD) was established as the concentration of each analyte that generated a response with a signal-to-noise ratio of 3. The precision was evaluated by calculating the coefficient of variation (CV) for multiplicate standard solutions ($n = 10$) prepared at three concentrations (0.1 ppb, 1 ppb and 10 ppb). To assess the influence of matrix effect, calibration curves were separately prepared in methanol and plasma. The matrix effect of each compound resulted from calculating the ratio between the slope of the calibration curve in plasma and the slope of the calibration curve in methanol. The recovery test was conducted by separately spiking 1 ppb of each compound in plasma followed by the procedures as mentioned in the following section. The analyzed concentrations were then compared with the amounts of standards spiked to calculate the recoveries for these compounds.

2.3. Study subjects

Cord blood samples were provided from the Taiwan Birth Panel Study (TBPS). Study subjects were pregnant women and their neonates recruited between April 2004 and January 2005 from one medical hospital in Taipei city, one area hospital, and two clinics in Taipei County. Before enrolling the study participants, protocols used in this study were approved by the Institutional Review Board of National Taiwan University Hospital and informed consents were obtained from all subjects before delivery to collect umbilical cord blood and prenatal questionnaires. For all the samples, we collected cord blood at delivery and separated it into whole blood, plasma and DNA. The plasma samples were stored at −80 °C until processed for pesticides analysis.

2.4. Online SPE-LC/HESI/MS/MS analysis

An online SPE system consisting of a Hitachi L-2100 pump (Hitachi High Technologies America, Schaumburg, IL, USA) and a Hypersil GOLD C8 cartridge (20 mm × 2.1 mm, 1.9 μm, Thermo Scientific, Waltham, MA, USA) was used for sample cleanup. A Thermo Scientific Accela HPLC system with a quaternary pump, an autosampler and a Hypersil GOLD C18 analytical column (50 mm × 2.1 mm, 1.9 μm, Thermo Scientific, Waltham, MA, USA) was used for further separation. The automated analytical systems were connected by a six port divert valve on the mass spectrometer. The arrangement of the online SPE-LC/MS system (Fig. 1) was similar as that reported previously [16].

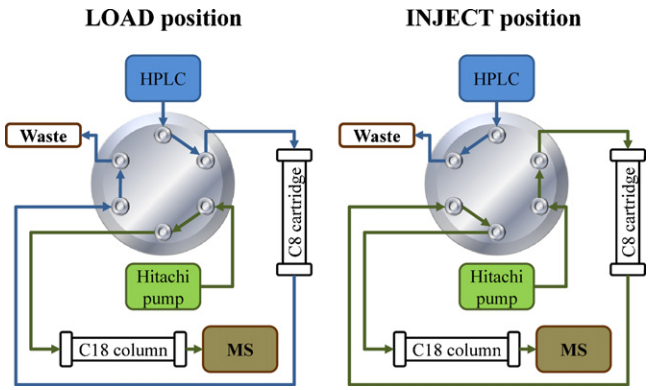

Fig. 1. Schematic diagram of online SPE-LC/MS system in two operating positions.

A Thermo Scientific TSQ Quantum Access mass spectrometer with a heated electrospray ionization (HESI) source was used for characterization and quantification. The selective reaction monitoring (SRM) mode was operated to monitor the ion mass transitions for each pesticide. An infusion study was performed by using a syringe pump directly connected to the ion source to provide a stable introduction of sample for method optimization. The mass spectra of chlorpyrifos and cypermethrin were obtained from infusion of 1 ppm of each pesticide at a flow rate of 10 μl/min and were optimized from stepwise parameter adjustment. The optimized parameters for the MS tune method were as follows: ion source polarity: positive ion mode; spray voltage: 3500 V; vaporizer temperature: 150 °C; sheath gas pressure (nitrogen): 50 (arbitrary units); auxiliary gas pressure (nitrogen): 15 (arbitrary units); and capillary temperature: 350 °C.

Three hundred microliters of methanol were added into 300 μl of cord blood plasma, followed by vigorous shaking and centrifugation at 12,000 rpm for 10 min. The supernatant was transferred into a sample vial and ready for instrumental analysis. One microliter of the pretreated sample was injected and delivered to a C8 cartridge for solid-phase extraction by the quaternary pump. The initial gradient of the mobile phase was held at 20% A (methanol) and 80% B (20 mM ammonium acetate aqueous solution) at a flow rate of 300 μl/min for 1 min, followed by a linear increase to 90% A from 1 to 1.5 min and held at 90% A from 1.5 to 5 min. The divert valve was switched from LOAD to INJECT position at 2.5 min, and the sample was eluted onto the analytical column by the Hitachi L-2100 pump with mobile phase C (10% 20 mM ammonium acetate aqueous solution:90% methanol mixture) at a flow rate of 300 μl/min. The divert valve was switched back to the LOAD position at 3.2 min to make up a transfer time of 0.7 min. The gradient of the mobile phase was returned to the initial condition from 5 to 5.1 min and the extraction cartridge was conditioned for 1.9 min before injection of the next sample. The run time cycle was 7 min for each sample.

3. Results and discussion

3.1. Method optimization

The $[M+H]^+$ ion for chlorpyrifos and the $[M+H]^+$ and $[M+NH_4]^+$ ions for cypermethrin at m/z 350, 416 and 433 are consistent with

reported studies [17–21]. Product-ion spectra of chlorpyrifos and cypermethrin were obtained and are shown in Figs. 2 and 3. The product ion spectrum obtained from *m*/*z* 350 of chlorpyrifos at 20 eV showed a fragment at *m*/*z* 294 consistent with fission of both the ethylethers on the phosphorothionate group (via the intermediate ion *m*/*z* 321) and the fragment at *m*/*z* 198 consistent with the aromatic moiety following loss of the phosphorothionate group (Fig. 2). The product ion spectrum of *m*/*z* 433 at 15 eV showed loss of ammonia to return the protonated parent at *m*/*z* 416 and the fragment at *m*/*z* 191 attributable to the dichlorovinyldimethylcyclopropane acylium ion from cleavage of the carbon–oxygen bond of the ester group (Fig. 3).

Two fragments derived from each precursor whilst using the same collision condition were chosen for confirmation, and the most abundant one was set as the quantification ion (Table 1). The event times for the 6-port divert valve on the mass spectrometer were set as follows. The mass spectrometer was first connected with the C8 cartridge to check the elution time and gradient of each pesticide. As shown in Figs. 4(a) and 5(a), the elution time of these two compounds was 2.72 min for chlorpyrifos and 2.93 min for cypermethrin. The mass spectrometer was then connected with the C18 column as shown in Fig. 1.

Table 1
SRM method parameters for the analysis of pesticides.

	Chlorpyrifos	Cypermethrin
Parent ion (*m*/*z*)	350	433
Qualification ion (*m*/*z*)	294	416
Quantification ion (*m*/*z*)	198	191
Collision gas pressure (Argon in mTorr)	1.5	1.5
Collision energy (V)	20	15
Tube lens	70	74

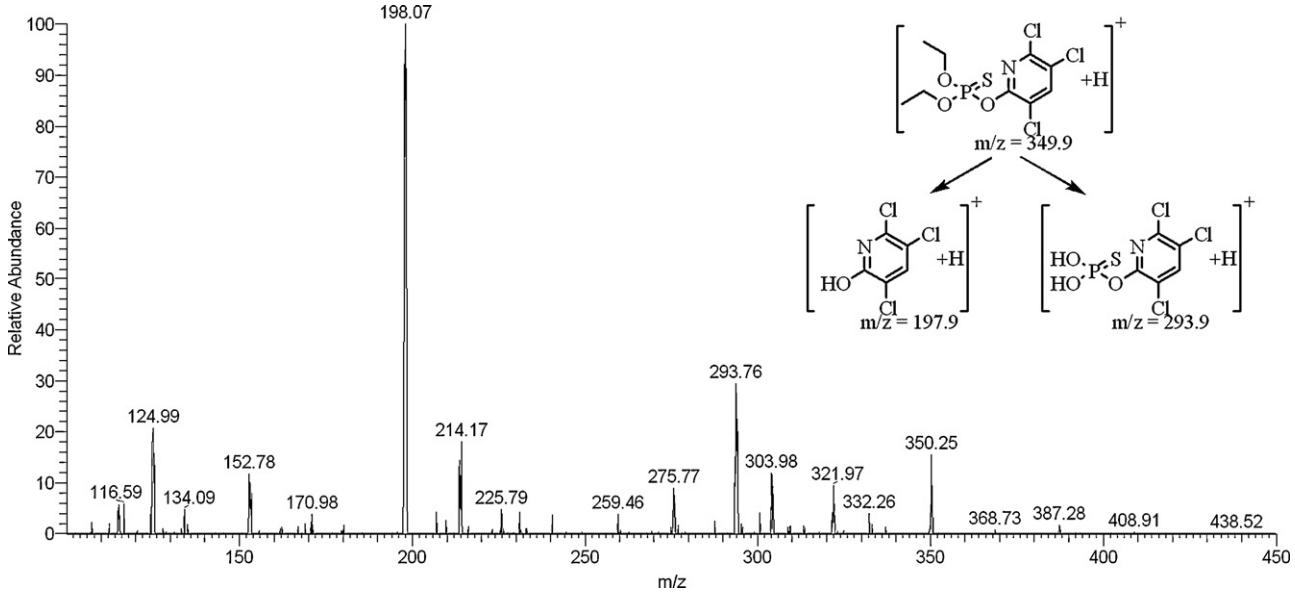

Fig. 2. Product ion spectrum of chlorpyrifos (*m*/*z* 350) at 20 eV demonstrated that the fragment at *m*/*z* 294 showing hydrolysis of ether ligands on phosphorothionate group of chlorpyrifos and the fragment at *m*/*z* 198 showing a loss of phosphorothionate group.

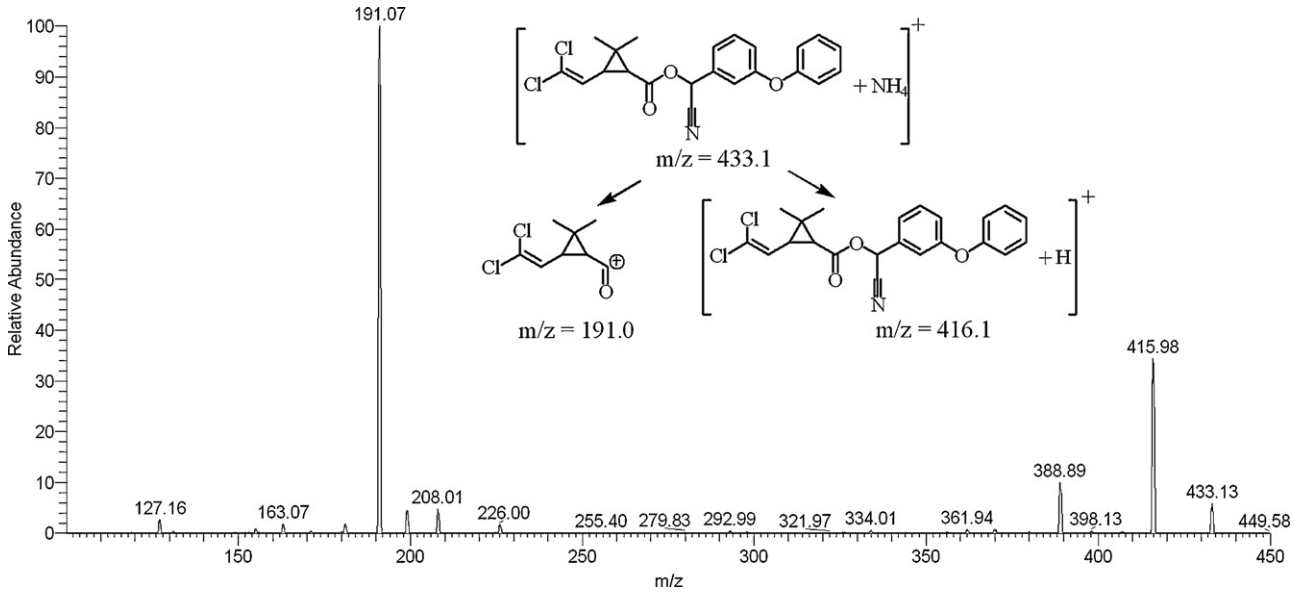

Fig. 3. Product ion spectrum of cypermethrin at 15 eV demonstrated that the fragment at *m*/*z* 416 as the $[M+H]^+$ ion of cypermethrin and the fragment at *m*/*z* 191 showing as the dichlorovinyldimethylcyclopropane acylium ion from cleavage of the carbon–oxygen bond of the ester group.

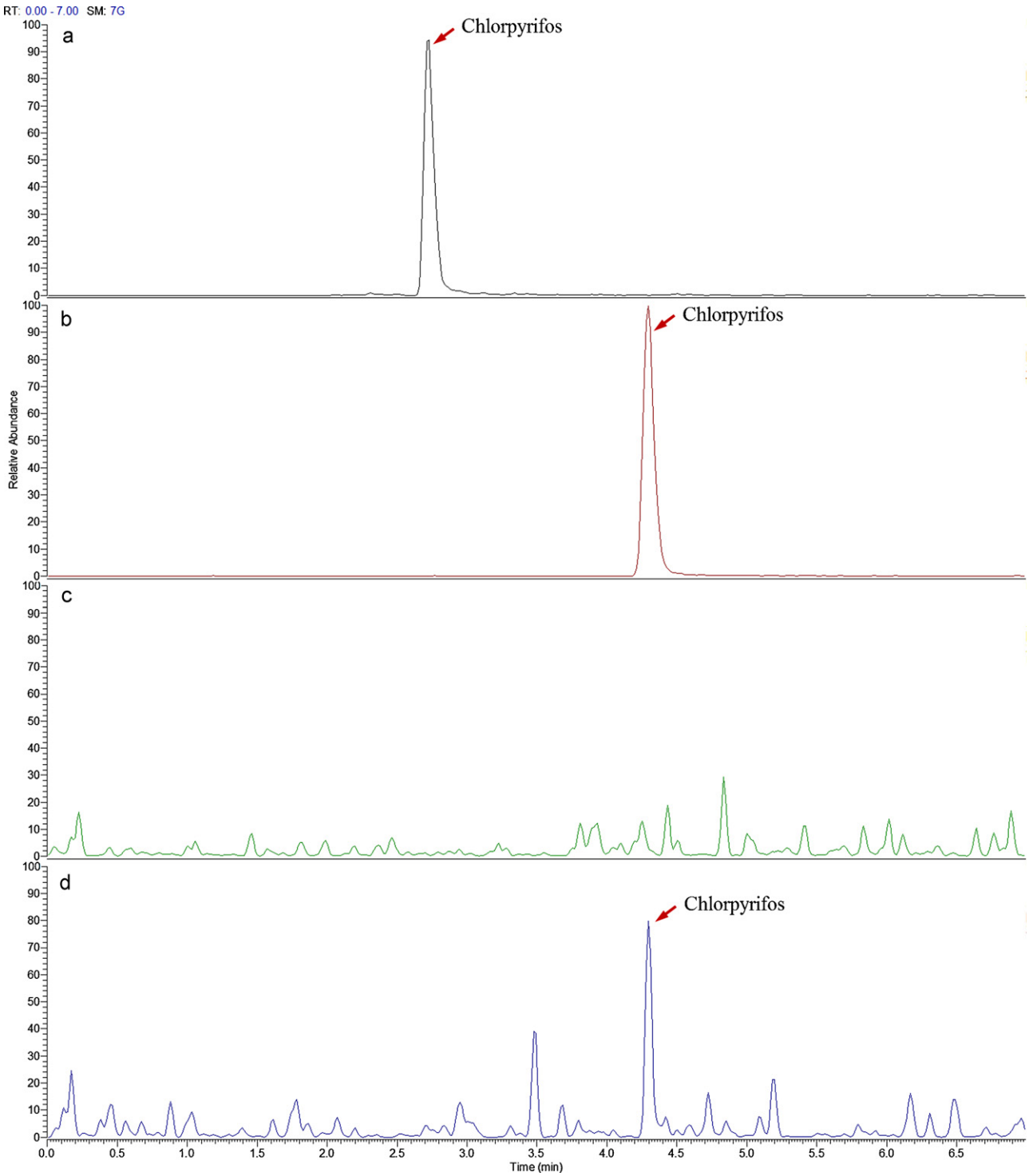

Fig. 4. Representative chromatograms of (a) 100 ppb of chlorpyrifos eluted from C8 cartridge only, (b) 100 ppb of chlorpyrifos eluted from C8 cartridge coupled with C18 column, (c) cord blood blank and (d) 0.1 ppb of chlorpyrifos spiked into cord blood.

The switching time of the divert valve was set at 2.5 min from LOAD to INJECT position and 3.2 min from INJECT to LOAD position respectively. The additional switching time of around 0.25 min on either end of the cutting time ensured that the peaks would not be lost due to retention time fluctuation. The analysis was then re-executed and the efficiency examined by comparing the abundance of corresponding peaks (Figs. 4(b) and 5(b)) from the C8 column directly and with the C18 column in series.

3.2. Method validation

The calibration curves showed excellent linearity ($r^2 > 0.998$) for both pesticides within the range of 0.1–100 ppb (Table 2). Analysis of the highest level of working standard was followed by injection of pure methanol, and no significant carryover effect was observed. Limits of detection (LODs) were 0.01 and 0.05 ppb at a signal-to-noise ratio of 3 for chlorpyrifos and cypermethrin respectively. The sensitivity of this method was comparable with that of

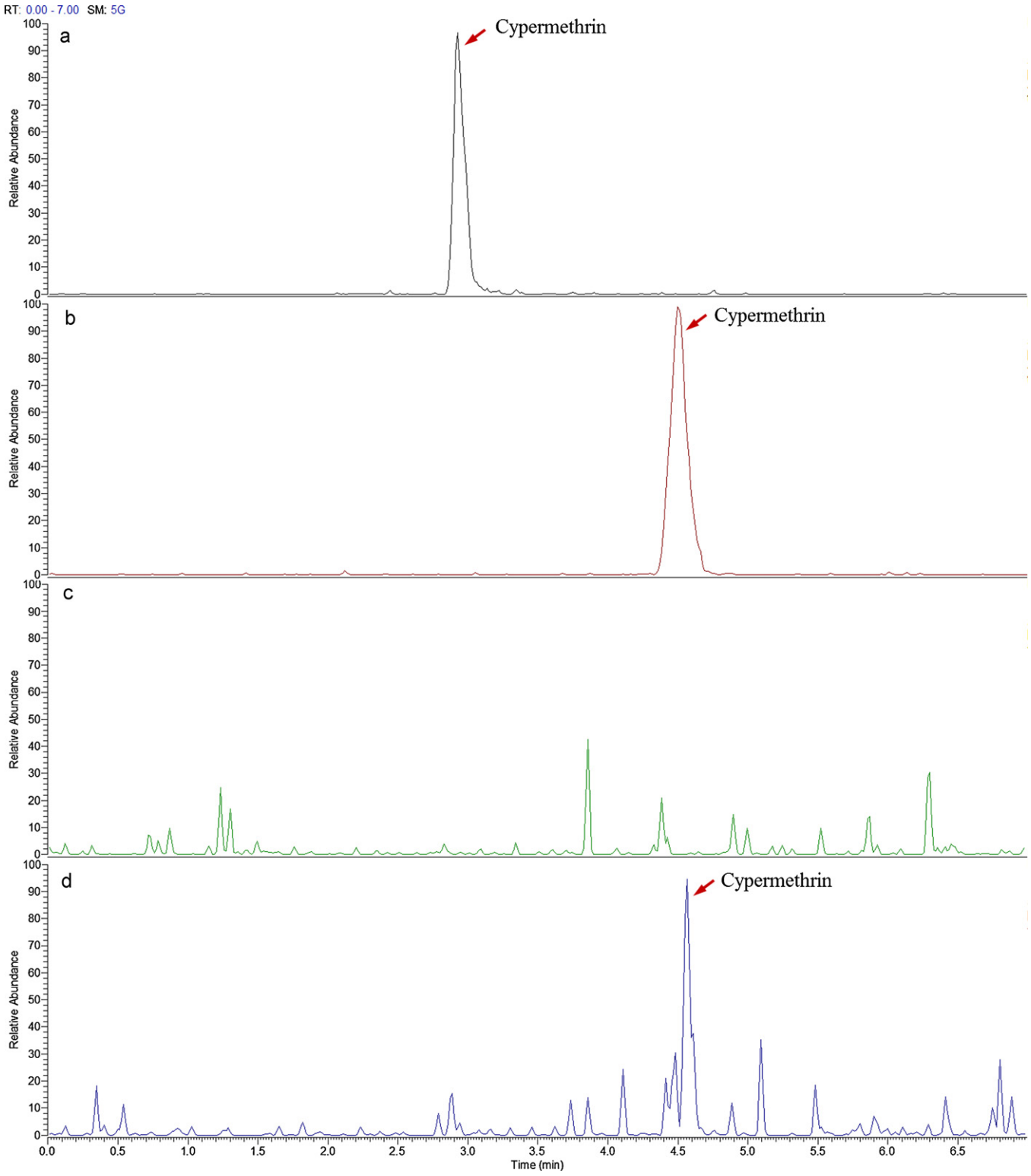

Fig. 5. Representative chromatograms of (a) 100 ppb of cypermethrin eluted from C8 cartridge only, (b) 100 ppb of cypermethrin eluted from C8 cartridge coupled with C18 column, (c) cord blood blank and (d) 0.1 ppb of cypermethrin spiked into cord blood.

other methods [15–18,20,21]. The chromatograms show no apparent interferences at the corresponding retention times for either pesticide (Figs. 4(c), (d) and 5(c), (d)). The precision expressed as the coefficient of variation (CV) was less than 12.2% based on analysis of multiplicate samples. Because the analysis is based on precipitated blood, the possibility that proteins or other macromolecules from the plasma might enhance or suppress the ionization of the analytes was examined. To estimate the influence of the matrix effect, calibration curves were separately prepared in methanol and plasma from 0.1 to 100 ppb. Table 2 summarizes the matrix effect of each analyte in plasma and shows that the responses of these analytes in plasma deviated from those in methanol by less than 5%. The recovery test samples were prepared by spiking with 1 ppb of each compound into cord blood plasma and analyzed using this

Table 2
Method validation data for the analysis of pesticides.

	Chlorpyrifos	Cypermethrin
Linear range (ppb)	0.1–100	0.1–100
Linearity (r^2)	0.9988	0.9993
LOD (ppb)	0.01	0.05
Precision (n = 10)		
0.1 ppb	12.0%	12.2%
1 ppb	10.4%	9.8%
10 ppb	8.8%	9.3%
Matrix effect	103.0%	95.7%
Recovery (n = 3)	97.2 ± 4.8%	93.5 ± 9.5%

Table 3
Concentration of pesticides (ppb) in umbilical cord blood plasma (n = 396).

	Mean	Minimum	25th percentiles	50th percentiles	75th percentiles	Maximum	DetNo[a]
Chlorpyrifos	0.38	<0.01	0.09	0.19	0.40	2.47	329
Cypermethrin	1.08	<0.05	<0.05	0.54	1.14	4.78	270

[a] DetNo, number of samples with pesticide concentrations above LOD.

SPE-LC/HESI/MS/MS method. The interpolated concentration for each analyte was then compared with the amount of standard spiked to calculate the recovery. As shown in Table 2, the recoveries in cord blood were 97.2 ± 4.8% and 93.5 ± 9.5% for chlorpyrifos and cypermethrin respectively.

3.3. Method application

Analysis of 396 cord blood samples showed 329 cases in which chlorpyrifos was detected and 270 incidences of cypermethrin. The results are summarized in Table 3. The mean concentrations of chlorpyrifos and cypermethrin in cord blood were 0.38 and 1.08 ppb respectively. Cypermethrin showed higher levels than chlorpyrifos in these cord blood samples. Because subjects were recruited from hospitals in Taipei city and county, and very few of them were involved in the use or production of cypermethrin and chlorpyrifos, it is reasonable to conclude that the presence of the substances can be attributed to dietary intake. The major exposure pathway in the domestic or urban population is likely to include residues in vegetables and fruits.

4. Conclusion

An online SPE-LC/HESI/MS/MS method was successfully developed to simultaneously analyze chlorpyrifos and cypermethrin in cord blood samples. Results of this study demonstrated comparable sensitivity and specificity of this method with previously published methods. In addition, this method simplified time-consuming sample pretreatment procedures, especially for cypermethrin, and so reduced the labor component of the analysis. The new method was particularly suitable for the high throughput methods needed to analyze a great number of samples in future epidemiology studies on the potential health effects caused by the exposure to chlorpyrifos and cypermethrin.

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