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Validation of a HRGC–ECNI/LRMS method to monitor short-chain chlorinated paraffins in human plasma

Jiazhi Xu[1,2], *Weijing Guo*[3], *Linhuan Wei*[3], *Yuan Gao*[1], *Haijun Zhang*[1,*], *Yichi Zhang*[1], *Ming Sun*[3], *Jiping Chen*[1,*]

1. *CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China. E-mail: xujiazhi@dicp.ac.cn*
2. *University of Chinese Academy of Sciences, Beijing 100049, China*
3. *Dalian Center for Disease Control and Prevention, Dalian 116021, China*

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ABSTRACT

Short-chain chlorinated paraffins (SCCPs) are produced in high volume and have the high potential to pose a threat to human health. However, little information is available for SCCP contamination in human blood/plasma/serum, mainly due to the difficulty of sample preparation and quantitative analysis. A method using high resolution gas chromatography coupled with electron capture negative ionization low resolution mass spectrometry (HRGC–ECNI/LRMS) was developed and validated to measure SCCPs in human plasma. The pretreatment process included protein denaturation and lipid elimination, liquid–liquid extraction with a mixture of *n*-hexane/dichloromethane (1:1, V/V), and cleanup on a multi-layer silica column. The blank controls, including procedural blank, vacuum blood collection tube blank, and instrumental blank, were the most pivotal points for the reliable analysis of SCCPs. The average value of procedural blanks was 9.0 ng/g; and the method detection limit (MDL), calculated as the sum of the average procedural blank value and 3 times of the standard deviation of the procedural blanks, was 12.6 ng/g plasma. The validated method was applied to measure the concentrations of the total SCCPs (∑SCCPs) in 50 plasma samples from a general population. The measured plasma concentrations of ∑SCCPs ranged from <MDL to 203 ng/g wet weight (ww), with an average value of 32.0 ng/g ww. The relative abundance profiles of SCCPs in plasma samples were dominated by C_{10}- and C_{11}-CP congener groups centered on Cl_{6-7}. The developed method can be used for the comprehensive and large-scale investigation of SCCP levels in human plasma.

Introduction

Chlorinated paraffins (CPs) are a group of polychlorinated *n*-alkanes that are widely used in commercial and industrial applications, such as metal cutting fluid, plasticizers and flame retardants in plastics and rubbers as well as sealants and leather conditioner (Fiedler, 2010). CPs are conventionally sub-classified into short-chain (C_{10-13}, SCCPs), medium-chain (C_{14-17}, MCCPs), and long-chain ($C_{>17}$, LCCPs) CPs based on carbon chain lengths (Wei et al., 2016). In China, most CPs were

* *Corresponding authors. E-mails:* hjzhang@dicp.ac.cn (Haijun Zhang), chenjp@dicp.ac.cn (Jiping Chen).

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produced as commercial mixtures, without being divided by carbon chain length. It was estimated that the production volume of SCCPs accounted for about 20% of the total CP yield, and the emission of SCCPs could reach 2560 tons in China in 2016 (Xu et al., 2014). Due to their persistence in environment, bioaccumulation, long-range transport potential and high toxicity, SCCPs have been listed in Annex A of the Stockholm Convention on Persistent Organic Pollutants (POPs) (UNEP, 2017).

SCCPs are highly toxic to many laboratory model organisms (Wei et al., 2016). Several studies have indicated the carcinogenic and mutagenic potential of SCCPs to thyroid, kidney and lymphoma cells in rats and mice (Myhr et al., 1990; Warnasuriya et al., 2010; Wyatt et al., 1993). The histopathological lesions and narcotic effects in juvenile rainbow trout liver and developmental toxicity in frog and zebrafish embryos after chronic exposure were also reported (Burýšková et al., 2006; Cooley et al., 2001). Recently, the endocrine-disrupting effect of SCCPs was indicated by exposure experiments with zebrafish and two kinds of cell lines (Liu et al., 2016; Zhang et al., 2016). In addition, our previous studies found that exposure to SCCPs could significantly affect glycerophospholipid metabolism, fatty acid metabolism, amino acid metabolism and purine metabolism in human hepatoma HepG2 cells and zebrafish embryos (Geng et al., 2015; Ren et al., 2018). These toxic effects imply that SCCPs have high potential to pose a threat to human health.

SCCPs have been ubiquitously found in various environmental matrices at relatively high level all over China (Liu et al., 2017; Wei et al., 2016; Wu et al., 2017; Xu et al., 2016; Zeng et al., 2017), and food intake and indoor dust ingestion were identified as the most important external exposure routes to SCCPs for general population (Gao et al., 2018). However, the data regarding the internal exposure to SCCPs for human are still far from being enough. Several studies reported the concentrations of SCCPs in human milks from Canada (Tomy, 1997), European countries (Reth et al., 2005a; SCA, 2012; Thomas et al., 2006), and Asia (Cao et al., 2017; Xia et al., 2017a, 2017b). Blood is an ideal matrix for judging the internal exposure of a pollutant to human considering it contacts with all body tissues and in equilibrium with organs (Smolders et al., 2009). However, the information on SCCP contamination in human blood/plasma/serum was scarce mainly due to the difficulties of sample preparation and quantitative analysis. Only Li et al. (2017) determined the levels of CPs in human blood using the reversed ultrahigh-pressure liquid chromatography coupled with chlorine-enhanced electron spray ionization-quadrupole time-of-flight mass spectrometry. The result indicated a significant occurrence of CPs in human blood from general population, and SCCPs showed a relatively higher accumulation (concentration range: 14–3500 ng/g blood, median value: 98 ng/g blood) compared with MCCPs (concentration range: 6.3–320 ng/g blood, median value: 21 ng/g blood) and LCCPs (concentration range: 1.0–21 ng/g blood, median value: 4.5 ng/g blood).

The high-resolution gas chromatography coupled with electron capture negative ionization low resolution mass spectrometry (HRGC-ECNI/LRMS) is still the most commonly used instrument in the routine detection of SCCPs as it exhibits the advantage of economical efficiency and convenience. In this study, based on HRGC-ECNI/LRMS analysis, a reliable method for the measurement of SCCPs in human plasma samples was developed by optimizing the sample preparation and implementing the stick quality assurance and quality control (QA/QC). The developed method is available for the comprehensive and large-scale investigation of SCCP contamination in human blood. The levels and congener group distribution of SCCPs in human plasma were also compared with other data reported for human blood and milk, determined by the high resolution mass spectrometry methods.

1. Materials and methods

1.1. Ethics statement

The experiments were approved by Dalian Center for Disease Control and Prevention and the utilization of human plasma was stood to guidelines of the Ethics Committee of Dalian Center for Disease Control (Dalian, China).

1.2. Standards, chemicals, and supplies

The standard stock solutions of the SCCPs (100 μg/mL in cyclohexane) with different chlorine content (51.5%, 55.5%, 63%) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The standard stock solutions of the SCCPs (100 μg/mL in cyclohexane) with chlorine content of 53.5% and 59% were obtained by 1:1 (V/V) mixing of the SCCPs solution with 51.5% and 55.5% chlorine content as well as with 55.5% and 63% chlorine content, respectively. 1,2,5,6,9,10-Hexachlorodecane ($C_{10}H_{16}Cl_6$, 98% purity) was synthesized by the addition reaction between 1,5,9-decatriene and trimethylchlorosilane in our laboratory. $^{13}C_6$-α-hexachlorocyclohexane ($^{13}C_6$-α-HCH) in *n*-nonane (100 μg/mL) was provided by Cambridge Isotope Laboratories (Andover, MA, USA). Silica gel (63–100 μm) was purchased from Sunchrom (Friedrichshafen, Germany), and anhydrous sodium sulfate from Damao (Tianjin, China). Silica gel was activated at 650°C for 4 hr prior to use. Acid silica gel and alkaline silica gel were prepared by mixing 100 g activated silica gel with 78.5 g concentrated sulfuric acid and 2 g potassium hydroxide (50 g/L in water), respectively, and then stored in desiccator before use. Anhydrous sodium sulfate was twice-washed using dichloromethane (DCM) by 30 min ultrasonic bath, and then dried at 300°C for 2 hr. The solvents for pesticide residue, i.e., *n*-hexane, DCM were obtained from J. T. Baker (Phillipsburg, USA). *n*-nonane (purity >99.0%, GC grade) was purchased from Fluka (Munich, Germany). Milli-Q water was used in the whole procedure.

1.3. Blood sample collection

The whole human blood samples stabilized with sodium citrate solution were collected by Dalian Center for Diseases Prevention and Control in November, 2015. After centrifugation, all the whole blood samples were stored at −80°C without separation of plasma until analysis.

1.4. Pretreatment procedure

The frozen blood samples were thawed in a refrigerator (4°C) and brought to room temperature, and then the plasma was immediately separated by centrifugation. Approximately

0.9–2.0 g of plasma was transferred to a pre-washed 50 mL glass centrifuge tube. Subsequently, 2 mL concentrated sulfuric acid was added into the glass tube, and then 10 mL *n*-hexane/DCM (1:1, *V/V*) was used as extraction solvent. The glass tube was then vortexed using a vortex mixer, following with centrifugation for 10 min at 4000 r/min before the organic phase was transferred to a flat-bottomed flask. After extraction, the extract was evaporated using a rotary evaporator until a volume of about 1 mL. The residual extract was then purified with a multilayer silica gel column. The column was packed with 5 g anhydrous sodium sulfate on the bottom, followed by 2% alkaline silica gel (2 g), activated silica gel (2 g), 44% sulfuric acid silica gel (5 g) and anhydrous sodium sulfate (6 g) on top. The first fraction of mobile phase *n*-hexane (50 mL) was discarded. The second fraction of mobile phase *n*-hexane/DCM (80 mL, 1:1, *V/V*) containing SCCPs was collected. The extract was concentrated and evaporated under a gentle stream of nitrogen at 40°C to near dryness, and then 10 μL of injection standard ($^{13}C_6$-α-HCH in *n*-nonane) was added for instrumental analysis.

1.5. Instrumental analysis

The analytical determination was processed using a TRACE GC ULTRA (Thermo Fisher Scientific, San Jose, USA) gas chromatography coupled with DSQ II (Thermo Fisher Scientific, San Jose, USA) mass spectrometry in electron capture negative ion mode. The chromatographic separation was performed using a fused silica capillary column of DB-5MS (15 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, USA). Helium (99.998%, Aga) and Methane (99.995%, Aga) were used as carrier gas and reagent gas with a constant flow of 0.8 mL/min and 2.0 mL/min, respectively. 1 μL of the final extracts was injected in splitless mode with the split outlet opening after 1.0 min. The injector temperature was set at 280°C. The oven temperature was initially 100°C for 2 min, raised by 20°C/min to 190°C, held for 2 min, and then heated to 310°C at 30°C/min and held for 10 min. The ion source and the interface temperatures were 200°C and 280°C, respectively. Analysis was performed in selected ion monitoring (SIM) mode, and the most and second most abundant isotopes of the $[M-Cl]^-$ ions of SCCPs and $^{13}C_6$-α-HCH were used as the quantitative and qualitative ions, respectively (Xu et al., 2016).

1.6. Method of identification and quantification

Retention time, mass charge ratios and peak shape with the respective retention time of the calibration standards were used as identification criteria. The quantification method described by Reth et al. (2005b) was used here with minor modifications. The relative total CP area was first normalized by the injection standard, and then the total response factor and the average chlorine content were calculated. The calibration curve was established between the total response factor for five SCCP standards and the calculated chlorine content. Five standard solutions of SCCP mixtures with different chlorine content (51.5%, 53.5%, 55.5%, 59%, and 63%) at a fixed concentration of 5 ng/μL, which give a similar response with the SCCPs in plasma samples, was used to yield five-point calibration curve.

1.7. QA/QC

In this study, a rigorous and systematic control of blanks was set up. The procedural blanks were first performed. A clean glass centrifuge tube was filled with 2 mL of Milli-Q water, and then extraction, cleanup and instrumental analysis were carried out as above described. The procedure blank reflects the possible contamination by glassware, materials and laboratory environment, and also by operators. Moreover, the vacuum blood collection tube blanks were also performed to assess the possible contamination from sample collection. A new vacuum blood collection tube containing 0.5 mL of sodium citrate solution was filled with 5 mL of Milli-Q water and equilibrated for one month, and then SCCPs were analyzed. An instrumental blank, so called "solvent blank" was implemented every day before the injection of plasma samples or standards. Solvent *n*-hexane was used as blank solvent, and the injection of blank solvent were continued until no-detection.

To minimize the risk of contamination, the utensil used in this study was restricted to glass, and aluminum foil was used as underlay when the screw lid is plastics. All equipment was thoroughly rinsed with *n*-hexane before experiment. Meanwhile, the whole sample preparation procedure was completed in one day in order to reduce the contamination from the ambient environment as possible.

The recovery rates of SCCPs in plasma were investigated by adding 50 ng, 100 ng and 250 ng of SCCP mixture standards (55.5% chlorine content) into 2.0 g plasma from plasma pools, respectively. The plasma pools used here were pre-extracted until no-detection of SCCPs, and stored at room temperature for 36 hr after SCCP standard addition. The plasma samples spiked with SCCP standards were extracted three times, and then cleaned up by multilayer silica gel column before instrument analysis. Moreover, a calibration standard solution containing 50 ng/mL $^{13}C_6$-α-HCH and 410 ng/mL $C_{10}H_{16}Cl_6$ was run daily to check the instrument calibration.

2. Results and discussion

2.1. Extraction efficiency

A total of 30 plasma samples were incorporated to form a plasma pool for optimizing the extraction condition. Approximately 3.0 g of plasma was taken out from the plasma pool and then added into a glass centrifuge tube. 10 mL of *n*-hexane/DCM (1:1, *V/V*) was used to extract SCCPs in plasma. The centrifugation was conducted after mixing homogeneously by a vortexer. The extraction efficiency of the total SCCPs (∑SCCPs) was limited by the frequency and duration of vortex process. The influence of extraction duration on extraction efficiency was first investigated when the extraction frequency was fixed to be 3 times, and the result was shown in Fig. 1a. When each extraction time was 5 min, the concentration of ∑SCCPs in plasma were determined to be (20.9 ± 1.2) ng/g wet weight (ww), which was very near to those ((19.2 ± 1.7) ng/g ww) determined when each extraction time was 10 min, whereas much higher than those ((6.5 ± 0.5) ng/g ww) determined when each extraction time was 2 min. This result suggested that the

optimized parameter was 5 min for each extraction. The effect of extraction frequency on extraction efficiency was further investigated when each extraction time was fixed to be 5 min. After extraction for 3 times, the concentration of ∑SCCPs in plasma were determined to be (21.6 ± 2.5) ng/g ww, which was almost similar to those ((21.6 ± 2.3) ng/g ww) determined after extraction for 5 times, whereas significantly higher than those ((14.7 ± 1.7) ng/g ww) determined after extraction for 2 times (Fig. 1b). This result indicated that extraction for 3 times could achieve a satisfactory extraction efficiency of SCCPs from plasma.

2.2. Clean-up efficiency

Plasma is a kind of bio-sample that is not as complicated as environmental samples such as soil and sediment, and thus we simplified the cleanup procedure of SCCPs. Only a multi-layer silica column was adopted to conduct the sample cleanup. In order to neutralize the possible acid residue from the extraction step, an alkaline silica gel layer was inserted into the multi-layer silica column. An aliquot of SCCP mixture standard (100 ng/μL, 55.5% chlorine content) was added onto the top of the multi-layer silica column, and then continuously eluted by *n*-hexane, *n*-hexane/DCM (1:1, *V/V*) and *n*-hexane/DCM (1:2, *V/V*). The recovery rate of ∑SCCPs in each fraction is expressed as percentage. As shown in Fig. 2, only 0.4% of ∑SCCPs were eluted by the first 50 mL *n*-hexane fraction. Subsequently, 50 mL of *n*-hexane/DCM (1:1, *V/V*) fraction eluted 99% of ∑SCCPs, and the following addition of 30 mL *n*-hexane/DCM (1:1, *V/V*) eluted 0.2% of ∑SCCPs. The recovery rate of SCCPs in the fraction of 50 mL *n*-hexane/DCM (1:2, *V/V*) was only 0.5%. These results indicated that the elution of 80 mL *n*-hexane/DCM (1:1, *V/V*) fraction could ensure an almost complete recovery of SCCPs from the plasma extract. In view of the extremely low recovery rates of SCCPs in *n*-hexane fraction and DCM/*n*-hexane (2:1, *V/V*) fraction, the eluting solution of the first 50 mL *n*-hexane fraction was discarded, and DCM/*n*-hexane (2:1, *V/V*) elution step was omitted.

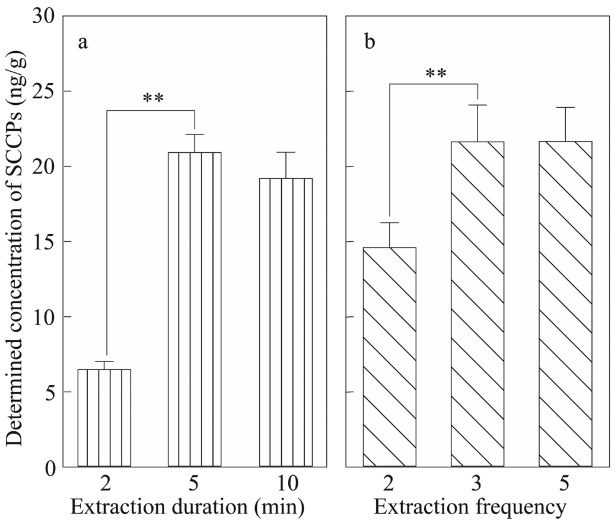

Fig. 1 – The determined concentrations of ∑SCCPs in plasma after extraction for different duration (a) and different frequency (b). **Significant difference at $p < 0.01$ level. SCCPs: Short-chain chlorinated paraffins.

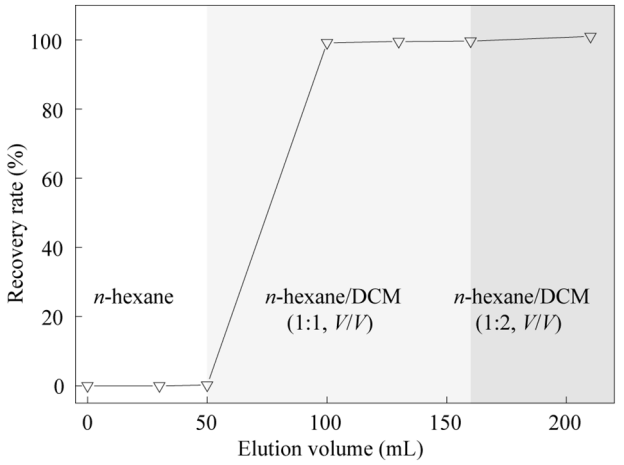

Fig. 2 – Elution curve of SCCPs (55.5% chlorine content) on the multi-layer silica column.

The plasma extract was used to further investigate the performance of optimized cleanup process of SCCPs. After cleanup, 10 μL of injection standard ($^{13}C_6$-α-HCH) was added for instrumental analysis. As shown in Fig. 3, the shape and retention time of chromatographic peak of SCCPs from plasma were almost identical to those of SCCP mixture standard (55.5% chlorine content), suggesting that the optimized cleanup process can be successfully applied to the plasma pre-treatment procedure.

2.3. Blanks

SCCPs have been ubiquitously found in the indoor environment, usually with a relatively high level in indoor airs (Shi et al., 2016; Wei et al., 2016), and therefore the procedural blanks showing the possible contamination must be conducted with every

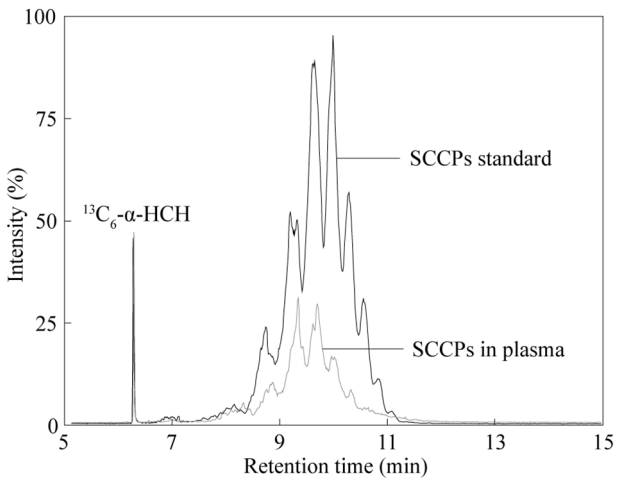

Fig. 3 – Total ion chromatograms of SCCPs in plasma and SCCP mixture standard (55.5% chlorine content).

sample set. In this study, a total of 10 procedural blanks were performed. The measured concentrations of ∑SCCPs ranged from 6.5 to 14.5 ng/g, with an average value of 9.0 ng/g.

The human serum was stored in plastic vacuum blood collection tubes containing sodium citrate solution as the anticoagulant. In order to assess the possible contamination by sample collection and storage, 4 vacuum blood collection tube blanks were performed. It was found that the concentration range (11.7–12.6 ng/g) of ∑SCCPs in vacuum blood collection tube blanks fell within those (6.5–14.5 ng/g) in procedural blanks. This result suggested that the contamination by vacuum blood collection tubes could be almost ignored.

The procedural blanks were used to evaluate the method detection limit (MDL) of ∑SCCPs in human plasma as following equation:

$$MDL = \overline{X_{blank}} + 3S_{blank} \quad (1)$$

where, $\overline{X_{blank}}$ (ng/g) and S_{blank} are the average concentration and standard deviation of ∑SCCPs in procedural blanks, respectively. According to Eq. (1), the MDL value of ∑SCCPs in human plasma was calculated to be 12.6 ng/g ww.

2.4. Method recovery and precision

Recovery rate of SCCPs from human plasma was determined by spiking SCCP mixture standard into plasma pool, in which SCCPs has been extracted until no detection. The recovery rate was calculated according to the ratio of the measured concentration to actual concentration of ∑SCCPs in the spiked plasma pool. Three levels (50, 100 and 250 ng) of SCCP mixtures (55.5% chlorine content) were spiked into 2.0 g plasma. After cleanup, SCCPs in plasma were analyzed. The calculated recovery rates of ∑SCCPs were in the range of 95%–115%, with an average value of 105%. In view of the higher recovery rate, the concentration of ∑SCCPs in plasma samples was not corrected by the average recovery rate. The method precision was also evaluated by quantifying SCCPs in these plasma samples spiked with SCCP mixture (55.5% chlorine content) standard. The relative standard deviations (RSDs) of SCCP concentrations in plasma samples spiked with 50, 100 and 250 ng were determined to be 10.9% (n = 3), 7.5% (n = 3), 3.1% (n = 3), respectively.

2.5. Levels and congener group profiles of SCCPs in plasma

The developed method was used to measure the concentrations of ∑SCCPs in 50 plasma samples, and the results according to age and gender are shown in Table 1. The procedural blank value was not deducted from the measured concentrations. SCCPs were detectable in 49 plasma samples, with the total concentrations ranged from 13.0 to 203 ng/g ww. The average level and the median value of ∑SCCPs in all plasma samples were 32.0 and 26.4 ng/g ww, respectively. The measured median concentrations of ∑SCCPs in human plasma in this study were lower than those in human blood samples (range: 14–3500 ng/g, median value: 98 ng/g) measured by ultrahigh-pressure liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (Li et al., 2017). The highest concentration of ∑SCCPs (203 ng/g ww) was found in the plasma samples from old female group, and the average concentration of ∑SCCPs in plasma samples of old age group (age: 60–88) (41.6 ng/g ww) was obviously higher than those of middle age group (age: 40–59) (21.8 ng/g ww) and young age group (age: 10–39) (31.3 ng/g ww). Nevertheless, the plasma concentrations of ∑SCCPs did not exhibit significant differences between old age group and middle age group as well as between old age group and young age group. In addition, no statistical difference was observed for the plasma concentrations of ∑SCCPs between males and females.

In order to compare our results with the concentrations of ∑SCCPs in human milk reported by other studies, the concentration unit of ∑SCCPs in human milk reported by other studies was transformed from ng/g lipid to ng/g wet weight (ww). The lipid contents were assumed to be 3.5% if there is no lipid content data available (SCA, 2012; Xia et al., 2017a, 2017b). The reported concentration of ∑SCCPs in human milk were in the ranges of 1.5–4.7 ng/g ww (average, 3.3 ng/g ww) in Swedish (SCA, 2012), 1.7–28.7 ng/g ww (average, 8 ng/g ww) in the UK (Thomas et al., 2006), <MDL –515.2 ng/g ww (average, 36.9 ng/g ww) in China (Cao et al., 2017; Xia et al., 2017a, 2017b). The measure plasma concentrations of ∑SCCPs (range: <MDL –203 ng/g ww, average: 32.0 ng/g ww) in this study were obviously higher than the reported concentrations of ∑SCCPs in human milk from Europe, whereas comparable to those in human milk from China.

The distribution profiles of SCCP congener groups were presented according to the relative abundance of monitored ions. As shown in Fig. 4, C_{10}-CPs and C_{11}-CPs predominated over the distribution profiles of SCCP congener groups in plasma samples. $Cl_{6–8}$-CPs showed the higher relative abundances in the congener groups C_{10}- and C_{11}-CPs, while $Cl_{5–7}$-CPs had the higher relative abundances in the congener groups C_{12}- and C_{13}-CPs. The carbon and chlorine congener group revealed here were similar with those in Chinese human milk which

Table 1 – Levels of ∑SCCPs in human plasma samples together with age and gender.

Age	Gender	n	Concentration range (ng/g ww)	Average [b] (ng/g ww)	Median value (ng/g ww)
10–39	Male	10	22.6–54.0	33.7	30.8
	Female	6	20.3–32.1	27.3	29.1
40–59	Male	10	14.2–32.2	22.6	21.8
	Female	6	13.1–28.8	20.4	18.8
60–88	Male	9	20.2–102.6	34.2	27.0
	Female	9	<MDL [a]-203	49.1	25.6
Total		50	<MDL-203	32.0	26.4

[a] Method detection limit.
[b] the concentration below MDL was treated as 0.5MDL when the average values were calculated.

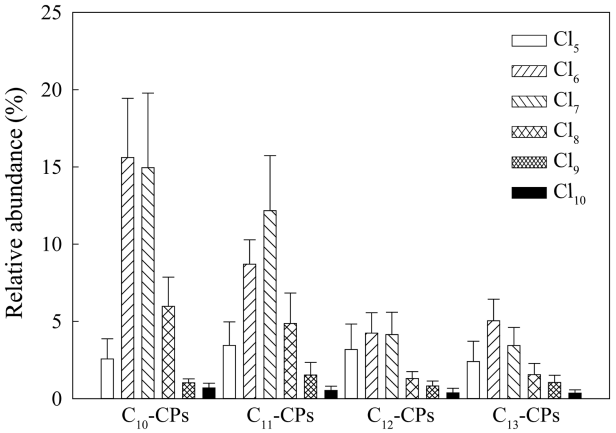

Fig. 4 – Congener group profiles of SCCPs in 49 plasma samples.

determined by a high resolution mass spectrometry method (Xia et al., 2017a, 2017b). The carbon congener group profile determined in this study was also consistent with those in aquatic invertebrates, fishes and terrestrial avian muscles of China (Luo et al., 2015; Ma et al., 2014; Zeng et al., 2017; Zhou et al., 2018). The similar congener patterns should result from the same bioaccumulation behaviour of SCCPs in human body and biota, which decisively depend on the chemical physical properties of SCCPs such as K_{ow} (Huang et al., 2017).

3. Conclusions

In this study, a HRGC-ECNI/LRMS method was developed and validated for the reliable analysis of SCCPs in human plasma. Mixed solvent of *n*-hexane/DCM (1:1, *V/V*) was used to extract SCCPs in plasma. The optimized extraction frequency and duration were 3 times and 5 min for each time extraction. The procedural blanks showing the possible contamination was conducted with every sample set. The MDL value was calculated to be 12.6 ng/g ww. The sample cleanup was conducted on a multi-layer silica column, by which most interferents for SCCP analysis could be removed, and the elution of 80 mL *n*-hexane/DCM (1:1, *V/V*) fraction ensured an almost complete recovery of SCCPs from the plasma extract. Based on the validated method, a survey of SCCP levels in human plasma samples from a general population was conducted. Among 50 plasma samples, SCCPs were detectable in 49 plasma samples. The measured plasma concentrations of ∑SCCPs ranged from <MDL to 203 ng/g ww, with an average value of 32.0 ng/g ww. The significant occurrence of SCCPs in Chinese human plasma suggests that regulatory actions are needed to reduce the emission and human exposure of SCCPs in China.

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