Elsevier Editorial System(tm) for Journal of Chromatography A

Manuscript Draft

Manuscript Number: JCA-20-536R1

Title: Optimization of hollow-fiber liquid phase microextraction for polychlorinated biphenyls in human breast milk

Article Type: Full length article

Keywords: Hollow Fiber Liquid Phase Microextraction, breast milk, Polychlorinated Biphenyls, multivariate optimization, gas chromatography

Corresponding Author: Professor TAMARA GARCIA-BARRERA, Professor

Corresponding Author's Institution: UNIVERSITY OF HUELVA

First Author: María del Carmen Villegas-Álvarez

Order of Authors: María del Carmen Villegas-Álvarez; Belén Callejón-Leblic; Gema Rodríguez-Moro; José Luis Gómez Ariza; Ana Arias-Borrego; TAMARA GARCIA-BARRERA, Professor

Abstract: A reliable and sensitive analytical approach has been optimized for the extraction of seven polychlorinated biphenyls (PCBs) from human breast milk. Hollow fiber liquid phase microextraction (HF-LPME) was applied for the first time for the extraction and pre-concentration of the analytes. Analytes were separated by gas chromatography with electron capture detector (GC-μECD) for the sensitive detection and mass spectrometry for the unequivocal identification. A rotable central composite design (RCCD) was performed for the multivariate optimization of the method. The best results were obtained at 40°C during 30 min and 600 rpm of stirring speed using a hollow fiber length of 5 cm and toluene as an extractant phase and salt addition was not required. The detection limits were in the range 7-14 ng L-1 for PCBs. The coefficients of determination of the calibration curves indicated good linearity (R2> 0.96) and the enrichment factors ranged from 74-143. This type of study is of great importance due to the deleterious effect that the presence of contaminants can produce in infants health related to the immature character of the defense system. Moreover, exclusive breastfeeding is recommended by neonatologists up to six months of life and as complementary food during the first two years.

Dear Editor,

Please find enclosed the amended version of the paper “Optimization of hollow-fiber liquid phase microextraction for polychlorinated biphenyls in human breast milk” by M.C. Villegas-Álvarez, B. Callejón-Leblic, G. Rodríguez-Moro, A. Arias-Borrego and T. García-Barrera, to be considered for publication in Journal of Chromatography A. The manuscript has been considerably improved taking into account all the points raised by the reviewers. A detailed point-by-point response to each comment is also attached.

The present paper describes for the first time the application of hollow fiber liquid phase microextraction (HF-LPME) for polychlorinated biphenyls (PCBs) in human breast milk. A new method has been optimized using multivariate approaches. The new method is very sensitive and reliable in comparison with other proposed in the literature.

Sincerely yours,

Tamara García Barrera

Highlights

- A HF-LPME method was developed for the determination of PCBs in human breast milk.
- The HF-LPME method is very simple since a sample preparation step is not required.
- A multivariate design (RCCD) was applied to optimize the extraction conditions.
- Extraction time, temperature and length of the fiber were main experimental factors.
- The proposed approach provides limits of detection in the range of 7-14 ngL^{-1}

Optimization of hollow-fiber liquid phase microextraction for polychlorinated biphenyls in human breast milk

M.C. Villegas-Álvarez[1,2], B. Callejón-Leblic[1,2], G. Rodríguez-Moro[1,2], J.L. Gómez-Ariza[1,2], A. Arias-Borrego[*1,2] and T. García-Barrera[*1,2]

[1]Research Center on Natural Resources, Health and the Environment (RENSMA). [2]Department of Chemistry "Prof. J.C. Vílchez Martín". University of Huelva. Fuerzas Armadas Ave., 21120 Huelva (Spain).

Corresponding authors at:

Department of Chemistry. Faculty of Experimental Science. University of Huelva. Campus de El Carmen. 21007, Huelva. Tel.: +34959219962, fax: +34 959 219942, e-mail address: tamara@dqcm.uhu.es (T. García-Barrera); Tel.: +34959219033, fax: +34 959 219942, e-mail address: ana.arias@dqcm.uhu.es (A. Arias-Borrego).

ABSTRACT

A reliable and sensitive analytical approach has been optimized for the extraction of seven polychlorinated biphenyls (PCBs) from human breast milk. Hollow fiber liquid phase microextraction (HF-LPME) was applied for the first time for the extraction and pre-concentration of the analytes. Analytes were separated by gas chromatography with electron capture detector (GC-μECD) for the sensitive detection and mass spectrometry for the unequivocal identification. A rotable central composite design (RCCD) was performed for the multivariate optimization of the method. The best results were obtained at 40°C during 30 min and 600 rpm of stirring speed using a hollow fiber length of 5 cm and toluene as an extractant phase and salt addition was not required. The detection limits were in the range 7-14 ng L^{-1} for PCBs. The coefficients of determination of the calibration curves indicated good linearity (R^2> 0.96) and the enrichment factors ranged from 74-143. This type of study is of great importance due to the deleterious effect that the presence of contaminants can produce in infants health related to the immature character of the defense system. Moreover, exclusive breastfeeding is recommended by neonatologists up to six months of life and as complementary food during the first two years.

Keywords: Hollow Fiber Liquid Phase Microextraction, breast milk, Polychlorinated Biphenyls, multivariate optimization, gas chromatography.

1. Introduction

Polychlorinated biphenyls (PCBs) are environmental pollutants with deleterious effects for humans, characterized by a high persistence and bioaccumulation capacity [1–3]. They are a class of persistent organic pollutants (POPs), considered a major public concern [4]. The existing 209 congeners contain from one to eight atoms of chlorine in different positions, which belong to poly-, mono- and non-ortho substituted PCBs. They are semivolatile, hydrophobic and have the basic unit of biphenyl, being structurally similar to thyroid hormones, which in turn led to thyroid disruption [5].

They were widely produced between 1930 and 1985 from industry, where they were used as plasticizers, insulating fluids in electric instruments and as sealants additives [6]. As a rule, the main source of PCBs are industries [7], leakage from landfills, incomplete incineration, inappropriate disposal, volatilization [7,8] and port activities [9,10]. They are considered as probable human carcinogens [2] by the Environmental Protection Agency (EPA) and as Group I human carcinogens by the International Agency for Research on Cancer (IARC) classified them [11]. During the prenatal period, exposure to PCBs comes from placental transfer and human breast milk (HBM) [12] causing health problems such as low birth weight [13], reduced head circumference [14], neurological effects [15] and thyroid disruption [5]. For this reason, several studies have suggested the use of breast milk as an indicator of the level of POPs in humans [16,17]. The content of lipids in breast milk is very high ranging from 3.5-4.5 per 100 g (mature milk) and due to the lipophilic character of polychlorinated biphenyls, they are accumulated and transferred to infants during breastfeeding [18].

For this reason, analytical methods for the sensitive and selective extraction, pre-concentration and determination of PCBs in this biological fluid are claimed. To this end, different analytical methods have been described for the extraction of PCBs from breast milk to be later analyzed by gas chromatography, like liquid-liquid extraction (LLE) [19], solid-phase microextraction (SPME) [20], accelerated solvent extraction (ASE) [21] and QuEChERS [16,18,22]. However, although nowadays hollow-fiber liquid phase microextraction (HP-LPME) is a very promising analytical technique, there is not any HP-LPME method described in the literature for the extraction of PCBs from breast milk. The most important advantages of HP-LPME are among others, the simplicity of operation and required equipment [23], low organic solvent consumption, robustness, low cost, disposable character (avoiding memory effects). The technique is based on the use of a supported liquid membrane containing the extractant phase and separated to the sample phase, which led to high surface area for the extraction and high enrichment factors [24].

After the extraction from breast milk, PCBs are usually determined by gas chromatography (GC) with mass spectrometry (MS) and/or electron capture detection (ECD) [16,18,22,25]. The sensitivity of ECD for halogenated compounds is usually better than MS detector, although this last detector allows the unequivocal identification of the compounds by mass spectra databases and tandem mass spectrometry [26].

In this paper, a sensitive and reliable HF-LPME method has been optimized and applied for the first time to the extraction of PCBs from human breast milk. The extracts have been analyzed by both GC-MS and GC-μECD for the determination of seven PCBs in breast milk. A multivariate optimization of the variables that affect the extraction was based on the combination and maximization of the response of all the PCBs. This optimization was performed using chemometric tools obtaining high enrichment factors. The developed method was sensitive, simple, reliable, reproducible and low-cost being easily transferable to routine analysis in laboratories to control the occurrence of PCBs in breast milk.

2. Experimental

2.1. Chemical and standard solution preparation

The mixture of PCBs standards (PCB# 1, 5, 29, 47, 116, 136,187,194, 206) were supplied by Sigma–Aldrich (Steinheim, Germany). Ultrapure water (18.2 MΩ cm) was obtained using a Millipore Milli-Q® Direct 8 equipment (Watford, UK). A solution with all the PCBs was prepared by dissolving in n-hexane the corresponding amount to attain the following concentrations of the standards (mg L^{-1}): PCB1 (100), PCB5 (100), PCB29 (10), PCB47 (10), PCB116 (10), PCB 136 (10) and PCB 187 (5). Intermediate working solutions used in direct injection were daily prepared by stepwise dilution of the above described solution with n-hexane. All the standard solutions were stored in darkness at 4ºC until analysis. All reagents and solvents used in this study were of the highest purity. N-hexane, acetone and toluene were supplied by Teknokroma (Barcelona, Spain). The hollow fiber membranes (Accurel Q3/2) were made of polypropylene (inner diameter 600 μm, wall thickness 200 μm, pore size 0.2 μm) (Wuppertal, Germany).

2.2. Samples

Human breast milk samples were taken after 48 h of life and stored at −80°C until their analysis. Samples were collected at the Juan Ramón Jiménez Hospital, during 2017. Written informed consent was obtained from all the participants and the study was approved by a local ethical committee.

For analytical validation of the method, the sample phase was composed by 350 μL of human breast milk to a final volume of 7 mL with ultrapure water (18.2 MΩ cm).

2.3. Instrumentation

Extracts were analyzed into a gas chromatograph model 6890N coupled to a ^{63}Ni microelectron capture detector (GC-µECD) (Agilent Technologies, Hewlett Packard, Wilmington, DE, USA). Chromatographic separation was conducted on a DB-5 chromatographic column (30m x 0.25mm x 0.25 µm). The carrier gas was helium (99.999%, 1.2 mL min^{-1}). A volume of 1µl of the extracts was injected into the GC using the splitless mode (splitless time: 30 s). The injector temperature was set at 250ºC. The oven temperature was programmed at 75ºC for 1 min, then increased to 150ºC ramped to 30ºCmin^{-1}, to 180ºC with a ramp to 5ºCmin^{-1} held for 15 min, to 190ºC ramped to 5ºCmin^{-1} for 5 min, to 200ºC with a ramp of 5ºC for 3min, to 280ºC with a ramp of 5ºC held for 2.5 min and then raised by 50ºCmin^{-1} to 290ºC with 4.8 min hold time. Nitrogen was used as makeup gas for the ECD detector (60 mL min^{-1}, 99.999%). The ChemStation software package (version A0903) was used for data acquisition. The extracts were simultaneously analyzed on an ion-trap GC-MS model ITQ 900 (Thermo Fisher Scientific, Thermo Fisher Scientific Spa, Rhone, Milan, Italy). The analytical column and oven temperature conditions were same than that used for GC-µECD. The injector temperature was set at 250 ° C. The carrier gas was helium (1.3 mL min^{-1}). The temperature of the transfer line between the chromatograph and the mass detector was set at 280 °C. For the separation of the peaks, the temperature program of the oven was the same above described for the GC-µECD. The ionization of analytes in the mass spectrometer was carried out by electronic impact (EI) with electronic ionization source at 70 eV in selected ion monitoring mode (SIM). PCBs were identified by retention time matching with standards, NIST database and tandem mass spectrometry.

2.4. Extraction of PCBs by HF-LPME

The extraction procedures were optimized prior to the application. The U-shaped configuration of the fiber and two-phase mode were used for HF-LPME. The arrangement is shown in Figure 1. The fibers were cut into 5cm portions and then cleaned into an ultrasound bath with acetone during 10 min. Then the cleaned and air-dried fiber was inserted into one of the medical syringe needles. The extractant phase toluene (500µL) was loaded into the lumen of the membrane by the immersion of the fiber into a vial containing that solvent, avoiding incomplete filling and air bubbles. The second end of the fiber was inserted into other syringe needle. Both syringes were introduced through of the septum into a vial containing toluene during 1 min in order to fill the pores of the membrane with the extractant phase (SLM). Afterwards, the U-shaped fiber was introduced into a 10 mL glass vial with 7 mL of sample (sample phase). The extraction was carried out in direct immersion mode during 40 min at 36ºC and 600 rpm. After that, the extract

was collected from the lumen and poured into a 1.5 mL vial with an insert of 250 µL by separating one end of the fiber from the syringe. Finally, 10 µL were obtained from the fiber and 1µL was injected into GC-µECD/MS.

2.5. Optimization of hollow-fiber liquid phase microextraction

The most influential variables in HF-LPME were optimized for the simultaneous analysis of 7 PCBs in human breast milk. Firstly, a preliminary screening using a univariate optimization was carried out to explore the influence of the different variables as well as the range of values that should be optimized. The following variables were studied in this work: extraction solvent, temperature, stirring rate, ionic streghth, pH, fiber length and extraction time. After that, a Rotable Central Composite Design (RCCD) of second order was used for the optimization of the variables taking into account the inetarations between them. To this end, the data were processed with the MINITAB®Release 16 Statistical Software (State College, USA). The combined response was also optimized using some home-made programs written in QUICKBASIC.

3. Results and discussion

3.1 Screening of the critical variables in the extraction process

A univariate approach was applied to screen the main factors with significant effects on the HF-LPME extraction procedure. To this end, when one of the variables is modified, the other features were set at the following values: temperature (25ºC), extraction time (5 min), HF length (4 cm), immersion time (1min), pH (not adjusted) and sample volume (350µL). The screened factors have been matching with those selected in most of the previous works about the HF-LPME extraction process of organic compounds [27,28]. The changes in the peak areas of the PCBs were plotted against the variation of each factor optimized and analyzed to select the ranges for the multivariate optimization.

3.1.1. Selection of the extractant phase for the HF-LPME

Several considerations should be taken into account to select the solvent for the extractant phase to be used for the HF-LPME: (i) it should be compatible with the material of the fiber (polypropylene) and (ii) the solvent should be immiscible with water to form two phases for the extraction, (iii) the viscosity should be adequate to allow filling the membrane pores as well as the diffusion of analytes, (iv) low volatility to avoid losses during the extraction procedure [29].

Under these conditions, three organic solvents were checked to extract the target analytes (1-octanol, toluene and n-hexane). Figure 2 shows the enrichment factors for all the analytes using these solvents concluding that toluene is the most efficient extractant phase for the extraction. Longer times than 1 min to embed the pores of the HF do not enhance the enrichment factors.

3.1.2. Effect of salt addition

The ionic strength could influence the partition coefficients of the analytes when two immiscible phases are in contact, which in turn affects the extraction efficiency. The ionic strength could be increased by adding salt, which could enhance the enrichment factors of the analytes in the organic solvent [27,28,30–34]. To this end, different percentages of sodium chloride were assayed up to 10% (w/v), but any effect was detected. The reason maybe could be the low polar character of the PCBs compounds, which enhance the extraction and does not need the modification of the ionic strength [35]. This result is in agreement with our previous work [27].

3.1.3. Effect of agitation

The stirring speed is important to enhance the mass transfer of the analytes from the sample phase to the extractant phase, reducing also the extraction time. Thus, the stirring rate was increased up to 1200 rpm, and the results showed a progressive increase of the enrichment factors until 600 rpm (Figure 3). However, higher values of this variables decrease the reproducibility of the results, mainly due to the occurrence of air bubbles in the solution. Based on these findings, 600 rpm was used in further experiments.

3.1.4. Effect of pH

The effect of pH in the extraction of PCBs by HF-LPME was optimized in the range of 4-12 and the corresponding enrichment factors are shown figure 4. Acid conditions were achieved by adding 6M HCl and basic conditions with 1M sodium hydroxide. There was no considerable effect in the enrichment factors of the PCBs varying the pH in the range of 6-8, in which the sample pH is included. Thus, the sample pH was not adjusted for subsequent experiments.

3.3. Central composite design

A RCCD was created [28] to optimize the three most important variables: temperature (T), time (tm) and membrane length (L). The number of experiments of the full factorial design can be calculated by 2^k, where k is the number of variables. These eight experiments can be augmented with 2k star points, which are the vertices of a cross-polytope at the coordinate axes of the design. Finally, C central points are included as replicates. Thus, for three variables to optimize, the CCD consist of 17 experiments ($2^k + 2k + C$). The variables to be optimized has five coded

levels (−α,−1, 0, 1, α), where α= $2^{k/4}$(~1.68), the star arm (distance) from the center to a star point. The –α (minimum) and +α (maximum) define the range of parameters to be optimized. To avoid systematic errors the trials were randomized. Table 1 collect the trials included in the RCCD showing the real and codel levels. Table 2 shows the response (areas peak of the analytes) of the PCBs obtained by HF-LPME in the different trials for the optimization of the temperature, extraction time and membrane length. After that, a regresion analysis was performed to obtain reduced models of polynomial equations to calculate the response of the analytes using the studied variables (Table 3). A significant Student´s "t-test" was performed to obtain the regression coefficients b_k, $s(b_k)$. An effect is considered significative when t_k= $|bk|/s(bk)$ is greater than the tabulated t_{critic}, (υ,P), with υ degrees of freedom (υ= number of run − number of coefficients to be calculated), at a *P* confidence level of 95% (p-value > 0.05).

Table 1. Experiments performed in the design of the RCCD.

	Time min (X1)		Temperature °C (X2)		Length cm (X3)	
Run	Coded level	Real level	Coded level	Real level	Coded level	Real level
1	-1	20	-1	35	-1	4
2	1	40	-1	35	-1	4
3	-1	20	1	45	-1	4
4	1	40	1	45	-1	4
5	-1	20	-1	35	1	6
6	1	40	-1	35	1	6
7	-1	20	1	45	1	6
8	1	40	1	45	1	6
9	0	30	0	40	0	5
10	0	30	0	40	0	5
11	-1.68	13.2	0	40	0	5
12	1.68	46.8	0	40	0	5
13	0	30	-1.68	31.6	0	5
14	0	30	1.68	48.4	0	5
15	0	30	0	40	-1.68	3.32
16	0	30	0	40	1.68	6.68
17	0	30	0	40	0	5

Table 2. Peak areas of the analytes after the analysis by HF-LPME from the RCCD trials.

Run	PCB 1	PCB 5	PCB 29	PCB 47	PBC 116	PCB 136	PCB 187
1	1445437	151166	341421	2982558	992053	576920	1079141
2	1224615	315737	459125	2072237	2018372	1072200	1138348
3	2532024	978107	290671	144663	445166	1094476	684602
4	1514127	106678	381923	151113	739466	986393	670114
5	2090428	13532203	1635603	803381	471934	2719882	241383

6	1787659	18015125	1041421	873296	823271	2091914	385883
7	1981037	17599705	395431	754873	774625	1484487	533688
8	215935	10079523	249864	784821	1092053	1363427	421684
9	32821417	8971327	1635202	1906952	14896527	1270088	565888
10	47586625	9125733	1444278	1706678	13739257	1592765	542409
11	8670406	338390	810645	229711	236551	1098930	394146
12	10514127	13151374	1637315	1893551	21003809	1177669	498725
13	4670909	11119787	1980343	1935806	15188631	1962856	569321
14	5709354	1298494	455265	617351	749486	669072	431516
15	996262	145265	1044196	287919	1859647	645741	887167
16	8670406	15131803	753395	436889	1941386	1285688	565888
17	4670909	9599705	1867114	1383234	1305880	1221292	581988

As we can see in Table 3, the temperature (T) and the HF length (L) have the most significant effects in the response of all the target analytes (the variables with squared terms indicate a high effect in the response of the analyte). The extraction time also has a significant effect in the response of PCB116, as can be seen in its equation model. In almost all the trials, the interaction between the temperature (T) and HF length (L) enhance the peak areas of the PCBs.

Table 3. Mathematical models of responses and variables.

Model equation
$PCB1=285974-900493T^2-913135L^2$
$PCB5=917345+607208L$
$PCB29=168089-346052T-372945L^2$
$PCB47=165103-521151T+577754T*L+412916L^2$
$PCB116=103614+27027tm-186973T-65655L$
$PCB136=134154-271583T+366794L-299454T*L$
$PCB187=557634-185368L+148860T*L+77180L^2$

Equation model: $y=B_0+\Sigma B_0 Xi+\Sigma B_{ii}X_i^2+\Sigma B_{ij}X_iX_j$
Response (peak area)
Variables: *T (* temperature), *tm* (extraction time), *L* (HF length)

In order to have the compromise of the best response of each analyte according to the variables used, the responses of all the analytes were combined for a better optimization. In this way, the analytical operations are simplified and avoided possible rounding mistakes and without neglecting any term. The response variable, namely "Combined Response" is the weighed sum of all the responses using the equation (Eq.1):

$$COMB = \sum_i \left\{ kw_i \left| \frac{Y_i - G_i}{R_i} \right| \right\}$$

Where w_i is the weight of the variable, which can be chosen by the user (generally the unity), Y_i are the different responses for the optimization and R_i their range. When the aim is the maximization of the combined response, then G_i=minimum in the range (Y_i-1/2R_i) and K=1. The randomized optimization of the combined surface (tm, L, T) is carried out using a home-made algorism and the simultaneous optimum is the maximum of the COMB surface. Several previous studies have used this optimization method with very good results [27,36]. Figure 5 shows the 3D combined response and contour plots against the time, length and temperature as well as the optimum point. The optimum coded values were fixed at the following coordinates: temperature (T)=0.00, extraction time (tm)=0.00, and HF length (L)=0.00, which correspond to the decoded values of 40°C for the extraction temperature, 30 min for the extraction time and 5cm of length.

3.4. Method validation

For method validation, the figures of merit of the optimized HF-LPME GC-µECD method were calculated for the analysis of PCBs (Table 4) such as: coefficients of determination (R^2), linear range, limits of detection (LOD) and quantification (LOQ), relative standard deviation (%RSD) and enrichment factors (EF). The experiments were performed using a spiked sample phase (350 µL of human breast milk diluted with ultrapure water to a final volume of 7 mL). PCBs were under the detection limits in the samples used for method validation. The linearity was in the range 0.02 to 140 µg L^{-1} for PCB29 and PCB47, 0.05 to 70 µg L^{-1} for PCB187, and in the range of 0.03 to 140 µg L^{-1} for other PCBs determined, and the determination coefficients ranged from 0.96 to 0.98. Seven different concentration levels of the PCBs under study were prepared and analyzed to determine the LODs and LOQs. The limits of detection were calculated as the concentration corresponding to a peak area 3-fold higher than the background noise, and the limits of quantification as the concentration corresponding to a peak area 10-fold higher the background noise of the chromatogram (LOQ=10S/N). The LOD values ranged from 8 to 14 ng L^{-1} with the proposed method. These results are very low when compared with other methods previously published in literature Table 5. Under optimized HF-LPME conditions, the enrichment factors (EF), calculated as the ratio between the PCBs concentration in the extractant phase (Ca) and their initial concentration in the sample phase (Cd) [28,37], were satisfactory in the range of 74 to 143-fold that is in good agreement with other published works related with the use of HP-LPME for other food samples [27]. The results have confirmed that the features of the method are very good and the methodology can be successfully applied for the analysis of samples.

Table 4. Figures of merit of the HF-LPME/GC-μECD approach for the analysis of PCBs in human breast milk.

	Precursor ion m/z*	Recovery (%)	Repeatability RSD (%)[a]	Reproducibility RSD (%)[b]	EF	Range (ng L^{-1})	R^2	LOD (ng L^{-1})	LOQ (ng L^{-1})
PCB 1	188.08	73	5	7	124	30-1400	0.96	10	33
PCB 5	222.07	70	7	10	168	30-1400	0.98	8	27
PCB 29	256.05	82	6	9	128	20-1400	0.98	7	23
PCB 47	291.97	117	6	8	128	20-1400	0.97	8	27
PCB 116	325.98	97	9	11	144	30-1400	0.98	9	29
PCB 136	359.96	80	7	10	118	30-1400	0.97	10	33
PCB 187	395.88	72	4	7	75	50-700	0.97	14	47

[a]Intra-day precision, calculated by extracting n=5; [b]Inter-day precision, calculated by extracting n=5; *The compounds are unequivocally identified by GC-MS.

The optimized method has been applied to human breast milk for the analysis of a group of seven PCBs. In spite of human breast milk is a sample matrix very complex the proposed method is very simple because it does not include a sample preparation step, which is mainly due to the excellent clean-up capability of the HF-LPME technique. In fact, this procedure involves only an initial dilution of the sample in ultrapure water, followed by two-phase HF-LPME. To this end, the extraction recoveries (ER) were calculated using the sample phase fortified at three different concentrations (100 μg L^{-1} of PCB1 and PCB5, 5 μg L^{-1} of PCB187 and 10 μg L^{-1} of other PCBs). Recoveries obtained ranged from 70 to 109 % at those spiked level, which are in good agreement with other methods proposed in the literature (Table 4 and 5) [18,27,38]. For method precision evaluation, the reproducibility (inter-day) and repeatability (intra-day) were calculated using the same spiked sample phase than that for the extraction recoveries. Five replicates were analyzed in the same day for the repeatability and the results were very good ranging from 4 to 9 % for PCBs, when they are compared with other methods [18,27,38]. The reproducibility was calculated with five replicated analysis over 3 different days. The results were very good in the range of 7 to 11 % [18,27,38]. Figure 6 show the chromatograms obtained from a sample of human breast milk spiked with 10 μg L^{-1} of PCBs before and after the extraction using HF-LPME followed GC-μECD. As can be seen, the enrichment factors are very high, as well as the selectivity obtained after the accurate optimization of the HF-LPME combined with the high selectivity and sensitivity of the ECD detector for halogenated compounds.

3.5. Comparison of the developed method with current methods

Taking into account the extraction efficiency (enrichment factors) and sensitivity (LODs), the proposed approach was in good agreement with other methods for the extraction and preconcentration of the analytes from human breast milk. On the basis of the analytical features

collected in Table 4, the optimized method revealed that the LODs were very close to other analytical methods previously proposed in the literature, and the repeatability and (%RSD) the reproducibility (%RSD) were lower than those of other reported (Table 4 and 5). On the other hand, it is remarkable that the extraction time of the HF-LPME method is shorter than those of other methods. The comparison of the results revealed that the developed method is rapid, accurate and simple for the sensitive determination of PCBs in human breast milk samples.

Table 5. Comparison of parameters validation of the proposed method with others previously published for the extraction of PCBs in human breast milk.

Method	SPME [22]			QuEChERS [16,18]			
	LOD	LOQ	Recovery	LOD	Intra-day	Inter-day	Recovery
	(ngL^{-1})	*(ngL^{-1})*	*(%)*	*(ngL^{-1})*	*RSD (%)*	*RSD (%)*	*(%)*
PCB 1	-	-	-	-	-	-	-
PCB 5	-	-	-	-	-	-	-
PCB 29	1650	5000	83	200	4	11	96
PCB 47	2240	6790	99	200	14	10	102
PCB 116	1840	4330	101	100	9	9	107
PCB 136	1140	3470	101	100	7	10	104
PCB 187	1000	3040	96	50	8	11	101

4. Conclusions

In this work, a simple, effective and selective HF-LPME method has been accurately optimized for the analysis of PCBs from human breast milk, involving low consumption of organic solvents, high pre-concentration factors of the analytes and relatively low cost. This is the first time that HF-LPME is applied to breast milk samples for the extraction of PCBs, which are environmental pollutants with important deleterious effects in human health. A rotable second-order central composite multivariate design was applied to determine the operational variables which allows obtaining maximum peak areas of seven PCBs in human breast milk. The proposed method allows obtaining limits of detection in the range of 7-14 ngL^{-1} with average recoveries of 85% for the target analytes and enrichment factors very satisfactory when compared with previous analytical methods for the analysis of PCBs in breast milk. The proposed method can be easily automatizable, converted into a green analytical technique and transferrable to routine applications due to its low cost, sensitivity, simplicity, robustness and utility.

Acknowledgements

The authors thank projects UHU-1256905 (Operative program FEDER-Andalusia 2014-2020, Consejería de Economía, Conocimiento, Empresa y Universidad, Spain) and PGC2018-096608-B-C21 (Ministry of Science and Innovation, Spain). Authors also thank European Community (FEDER) for financial support (UNHU15-CE-3140 and UNHU13-1E-1611). The authors thank Dr. Eduardo García-Soblechero, Celia Pérez-Serralbo and Dr. Elisabeth Gómez-Santos, from the Pediatric Unit of Juan Ramón Jiménez Hospital for the collection of samples.

References

[1] A. Naqvi, A. Qadir, A. Mahmood, M. Baqar, I. Aslam, N. Jamil, M. Mumtaz, S. Saeed, G. Zhang, Screening of human health risk to infants associated with the polychlorinated biphenyl (PCB) levels in human milk from Punjab Province, Pakistan, Environ. Sci. Pollut. Res. In Press (2019). doi:10.1007/s11356-019-07126-2.

[2] US EPA 2015., Learn about Polychlorinated Biphenyls (PCBs) [Internet]., n.d. https://www.epa.gov/pcbs/learn-aboutpolychlorinated-%0Abiphenyls-pcbs.

[3] H. Arfaeinia, Z. Asadgol, E. Ahmadi, M. Seifi, M. Moradi, S. Dobaradaran, Characteristics, distribution and sources of polychlorinated biphenyls (PCBs) in coastal sediments from the heavily industrialized area of Asalouyeh, Iran, Water Sci. Technol. 76 (2017) 3340–3350. doi:10.2166/wst.2017.500.

[4] B. Cetin, S. Yurdakul, M. Keles, I. Celik, F. Ozturk, C. Dogan, Atmospheric concentrations, distributions and air-soil exchange tendencies of PAHs and PCBs in a heavily industrialized area in Kocaeli, Turkey, Chemosphere. 183 (2017) 69–79. doi:10.1016/j.chemosphere.2017.05.103.

[5] Z.-M. Li, M. Albrecht, H. Fromme, K.-W. Schramm, M. De Angelis, Persistent Organic Pollutants in Human Breast Milk and Associations with Maternal Thyroid Hormone Homeostasis, Environ. Sci. Technol. 54 (2020) 1111–1119. doi:10.1021/acs.est.9b06054.

[6] M.D. Erickson, R.G. Kaley, Applications of polychlorinated biphenyls, Environ. Sci. Pollut. Res. 18 (2011) 135–151. doi:10.1007/s11356-010-0392-1.

[7] N. Cardellicchio, A. Buccolieri, S. Giandomenico, L. Lopez, F. Pizzulli, L. Spada, Organic pollutants (PAHs, PCBs) in sediments from the Mar Piccolo in Taranto (Ionian Sea, Southern Italy), Mar. Pollut. Bull. 55 (2007) 451–458. doi:10.1016/j.marpolbul.2007.09.007.

[8] F. Smedes, J. De Boer, Determination of chlorobiphenyls in sediments - Analytical methods, TrAC - Trends Anal. Chem. 16 (1997) 503–517. doi:10.1016/S0165-9936(97)00080-0.

[9] S.H. Hong, U.H. Yim, W.J. Shim, J.R. Oh, Congener-specific survey for polychlorinated biphenlys in sediments of industrialized bays in Korea: Regional characteristics and

pollution sources, Environ. Sci. Technol. 39 (2005) 7380–7388. doi:10.1021/es050397c.

[10] S.H. Hong, U.H. Yim, W.J. Shim, D.H. Li, J.R. Oh, Nationwide monitoring of polychlorinated biphenyls and organochlorine pesticides in sediments from coastal environment of Korea, Chemosphere. 64 (2006) 1479–1488. doi:10.1016/j.chemosphere.2005.12.056.

[11] B. Lauby-Secretan, D. Loomis, Y. Grosse, F. El Ghissassi, V. Bouvard, L. Benbrahim-Tallaa, N. Guha, R. Baan, H. Mattock, K. Straif, Carcinogenicity of polychlorinated biphenyls and polybrominated biphenyls, Lancet Oncol. 14 (2013) 287–288. doi:10.1016/S1470-2045(13)70104-9.

[12] M.H.B. Müller, A. Polder, O.B. Brynildsrud, R. Grønnestad, M. Karimi, E. Lie, W.B. Manyilizu, R.H. Mdegela, F. Mokiti, M. Murtadha, H.E. Nonga, J.U. Skaare, A. Solhaug, J.L. Lyche, Prenatal exposure to persistent organic pollutants in Northern Tanzania and their distribution between breast milk, maternal blood, placenta and cord blood, Environ. Res. 170 (2019) 433–442. doi:10.1016/j.envres.2018.12.026.

[13] M.P. Longnecker, M.A. Klebanoff, H. Zhou, J.W. Brock, Association between maternal serum concentration of the DDT metabolite DDE and preterm and small-for-gestational-age babies at birth, Lancet. 358 (2001) 110–114. doi:10.1016/S0140-6736(01)05329-6.

[14] M.H.B. Müller, A. Polder, O.B. Brynildsrud, M. Karimi, E. Lie, W.B. Manyilizu, R.H. Mdegela, F. Mokiti, M. Murtadha, H.E. Nonga, J.U. Skaare, J.L. Lyche, Organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) in human breast milk and associated health risks to nursing infants in Northern Tanzania, Environ. Res. 154 (2017) 425–434. doi:10.1016/j.envres.2017.01.031.

[15] N. Ribas-Fitó, E. Cardo, M. Sala, M. Eulàlia de Muga, C. Mazón, A. Verdú, M. Kogevinas, J.O. Grimalt, J. Sunyer, Breastfeeding, exposure to organochlorine compounds, and neurodevelopment in infants., Pediatrics. 111 (2003) e580–e585. doi:10.1542/peds.111.5.e580.

[16] O.P. Luzardo, N. Ruiz-Suárez, M. Almeida-González, L.A. Henríquez-Hernández, M. Zumbado, L.D. Boada, Multi-residue method for the determination of 57 Persistent Organic Pollutants in human milk and colostrum using a QuEChERS-based extraction procedure, Anal. Bioanal. Chem. 405 (2013) 9523–9536. doi:10.1007/s00216-013-7377-0.

[17] I. Çok, B. Mazmanci, M.A. Mazmanci, C. Turgut, B. Henkelmann, K.W. Schramm, Analysis of human milk to assess exposure to PAHs, PCBs and organochlorine pesticides in the vicinity Mediterranean city Mersin, Turkey, Environ. Int. 40 (2012) 63–69. doi:10.1016/j.envint.2011.11.012.

[18] M. Pajewska-Szmyt, E. Sinkiewicz-Darol, U. Bernatowicz-Łojko, T. Kowalkowski, R. Gadzała-Kopciuch, B. Buszewski, QuEChERS extraction coupled to GC-MS for a fast

410 determination of polychlorinated biphenyls in breast milk from Polish women, Environ.
411 Sci. Pollut. Res. 26 (2019) 30988–30999. doi:10.1007/s11356-019-06201-y.
412 [19] V. Bencko, M. Černá, L. Jech, J. Šmíd, Exposure of breast-fed children in the Czech
413 Republic to PCDDs, PCDFs, and dioxin-like PCBs, Environ. Toxicol. Pharmacol. 18
414 (2004) 83–90. doi:10.1016/j.etap.2004.01.009.
415 [20] L. Röhrig, H.U. Meisch, Application of Solid Phase Micro Extraction for the rapid
416 analysis of chlorinated organics in breast milk, Fresenius. J. Anal. Chem. 366 (2000)
417 106–111. doi:10.1007/s002160050019.
418 [21] J. She, A. Holden, M. Sharp, M. Tanner, C. Williams-Derry, K. Hooper, Polybrominated
419 diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) in breast milk from the
420 Pacific Northwest, Chemosphere. 67 (2007) S307–S317.
421 doi:10.1016/j.chemosphere.2006.05.154.
422 [22] C.H. Kowalski, G.A. da Silva, R.J. Poppi, H.T. Godoy, F. Augusto, Neuro-genetic
423 multioptimization of the determination of polychlorinated biphenyl congeners in human
424 milk by headspace solid phase microextraction coupled to gas chromatography with
425 electron capture detection, Anal. Chim. Acta. 585 (2007) 66–75.
426 doi:10.1016/j.aca.2006.11.073.
427 [23] S. Pedersen-Bjergaard, K.E. Rasmussen, T. Grønhaug Halvorsen, Liquid-liquid
428 extraction procedures for sample enrichment in capillary zone electrophoresis, J.
429 Chromatogr. A. 902 (2000) 91–105. doi:10.1016/S0021-9673(00)00738-X.
430 [24] J. Lee, H.K. Lee, K.E. Rasmussen, S. Pedersen-Bjergaard, Environmental and
431 bioanalytical applications of hollow fiber membrane liquid-phase microextraction: A
432 review, Anal. Chim. Acta. 624 (2008) 253–268. doi:10.1016/j.aca.2008.06.050.
433 [25] B. Deng, J.Q. Zhang, L. Zhang, Y.S. Jiang, J. Zhou, D. Fang, H. Zhang, H.Y. Huang,
434 Levels and profiles of PCDD/Fs, PCBs in mothers' milk in Shenzhen of China:
435 Estimation of breast-fed infants' intakes, Environ. Int. 42 (2012) 47–52.
436 doi:10.1016/j.envint.2011.03.022.
437 [26] A.D. Nikolaou, T.D. Lekkas, S.K. Golfinopoulos, M.N. Kostopoulou, Application of
438 different analytical methods for determination of volatile chlorination by-products in
439 drinking water, Talanta. 56 (2002) 717–726. doi:10.1016/S0039-9140(01)00613-0.
440 [27] J. Manso, T. García-Barrera, J.L. Gómez-Ariza, New home-made assembly for hollow-
441 fibre membrane extraction of persistent organic pollutants from real world samples., J.
442 Chromatogr. A. 1218 (2011) 7923–35. doi:10.1016/j.chroma.2011.09.023.
443 [28] A. Domínguez-Tello, A. Arias-Borrego, T. García-Barrera, J.L. Gómez-Ariza,
444 Application of hollow fiber liquid phase microextraction for simultaneous determination
445 of regulated and emerging iodinated trihalomethanes in drinking water, J. Chromatogr.
446 A. 1402 (2015) 8–16. doi:10.1016/j.chroma.2015.05.020.

447 [29] M. Ghambarian, Y. Yamini, A. Esrafili, Developments in hollow fiber based liquid-
448 phase microextraction: Principles and applications, Microchim. Acta. 177 (2012) 271–
449 294. doi:10.1007/s00604-012-0773-x.
450 [30] S.-D.H. Pai-Shan Chen, Determination of ethoprop, diazinon, disulfoton and fenthion
451 using dynamic hollow fiber-protected liquid-phase microextraction coupled with gas
452 chromatography–mass spectrometry, Talanta. 69 (2006) 669–675.
453 [31] P.H. Howard, W.M. Meylan, Handbook of Physical Properties of Organic Chemicals,
454 CRC Press-Lewis Publishers, 1997.
455 [32] C. Basheer, R. Balasubramanian, H.K. Lee, Determination of organic micropollutants in
456 rainwater using hollow fiber membrane/liquid-phase microextraction combined with gas
457 chromatography-mass spectrometry, J. Chromatogr. A. 1016 (2003) 11–20.
458 doi:10.1016/S0021-9673(03)01295-0.
459 [33] C. Basheer, H.K. Lee, J.P. Obbard, Application of liquid-phase microextraction and gas
460 chromatography-mass spectrometry for the determination of polychlorinated biphenyls
461 in blood plasma, J. Chromatogr. A. 1022 (2004) 161–169.
462 doi:10.1016/j.chroma.2003.09.043.
463 [34] G.E.P. Box, K.B. Wilson, On the Experimental Attainment of Optimum Conditions, J.
464 R. Stat. Soc. Ser. B. 13 (1951) 1–38. doi:10.1111/j.2517-6161.1951.tb00067.x.
465 [35] M. Shamsipur, B. Hashemi, Extraction and determination of polycyclic aromatic
466 hydrocarbons in water samples using stir bar sorptive extraction (SBSE) combined with
467 dispersive liquid-liquid microextraction based on the solidification of floating organic
468 drop (DLLME-SFO) followed by, RSC Adv. 5 (2015) 20339–20345.
469 doi:10.1039/c4ra14959a.
470 [36] J.L. Gómez-Ariza, T. García-Barrera, F. Lorenzo, A.G. González, Optimisation of a
471 pressurised liquid extraction method for haloanisoles in cork stoppers, Anal. Chim. Acta.
472 540 (2005) 17–24. doi:10.1016/j.aca.2004.08.031.
473 [37] S. Ranjbar Banforuzi, M.R. Hadjmohammadi, Two-phase hollow fiber-liquid
474 microextraction based on reverse micelle for the determination of quercetin in human
475 plasma and vegetables samples, Talanta. 173 (2017) 14–21.
476 doi:10.1016/j.talanta.2017.05.058.
477 [38] G. Li, L. Zhang, Z. Zhang, Determination of polychlorinated biphenyls in water using
478 dynamic hollow fiber liquid-phase microextraction and gas chromatography-mass
479 spectrometry, J. Chromatogr. A. 1204 (2008) 119–122.
480 doi:10.1016/j.chroma.2008.07.081.
481
482

Legends of Figures

Figure 1. U-shaped HF-LPME configuration for two-phase extraction of PCBs from human breast milk.

Figure 2. Mean values (n=3) of the enrichment factors for the analytes using different solvents.

Figure 3. Mean values (n=3) of the enrichment factors for the analytes at different stirring speeds.

Figure 4. Mean values (n=3) of the enrichment factors for the analytes at different pH.

Figure 5. 3D combined response and contour plots of the surface shape against the temperature, time and length axis as well as the optimum point.

Figure 6. Chromatograms obtained from a human breast milk sample spiked with 10 µg L^{-1} of PCBs before (blue line) and after (red line) the extraction using HF-LPME followed GC-µECD.

Figure 1

Click here to download high resolution image

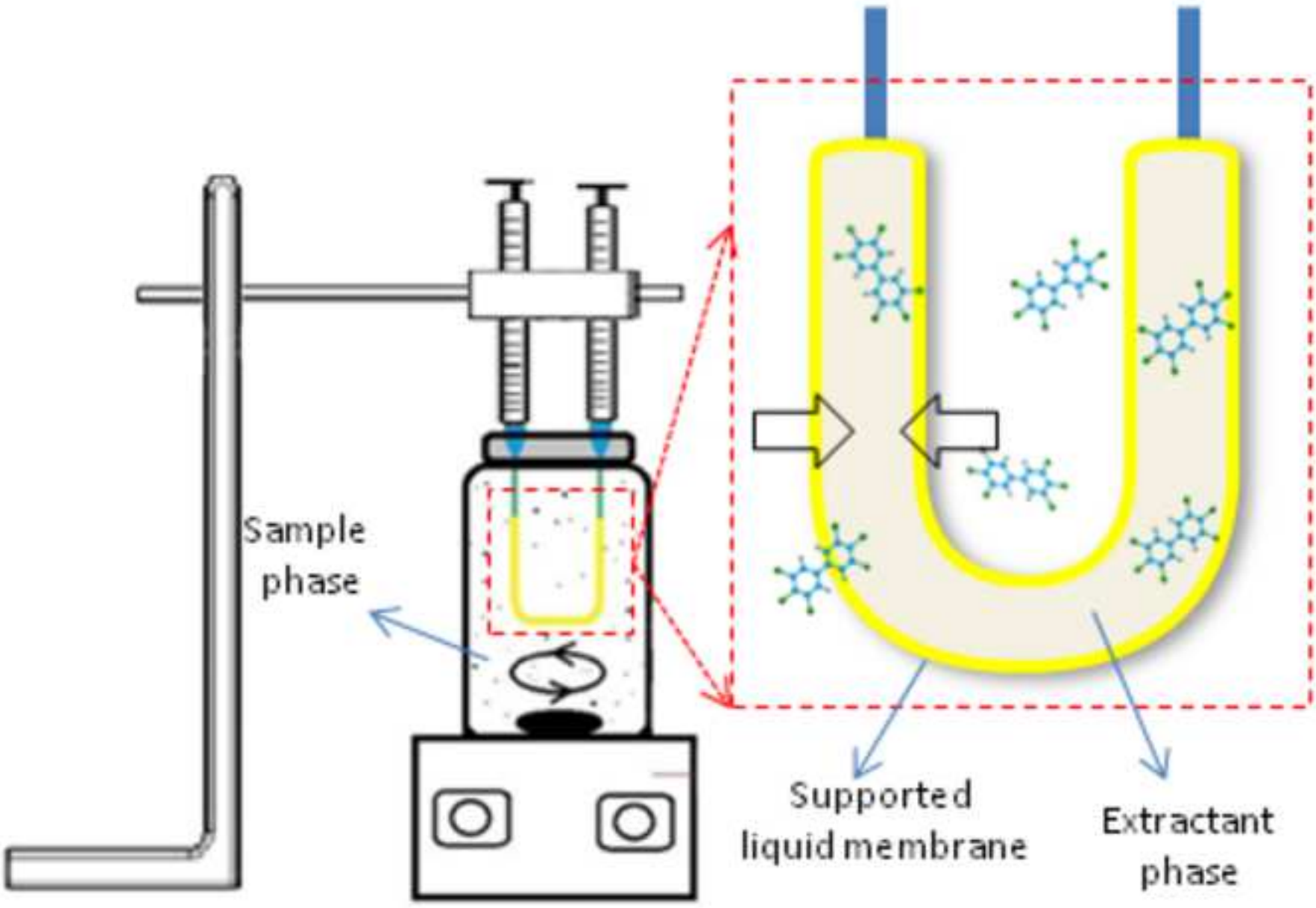


Figure 2

Click here to download high resolution image

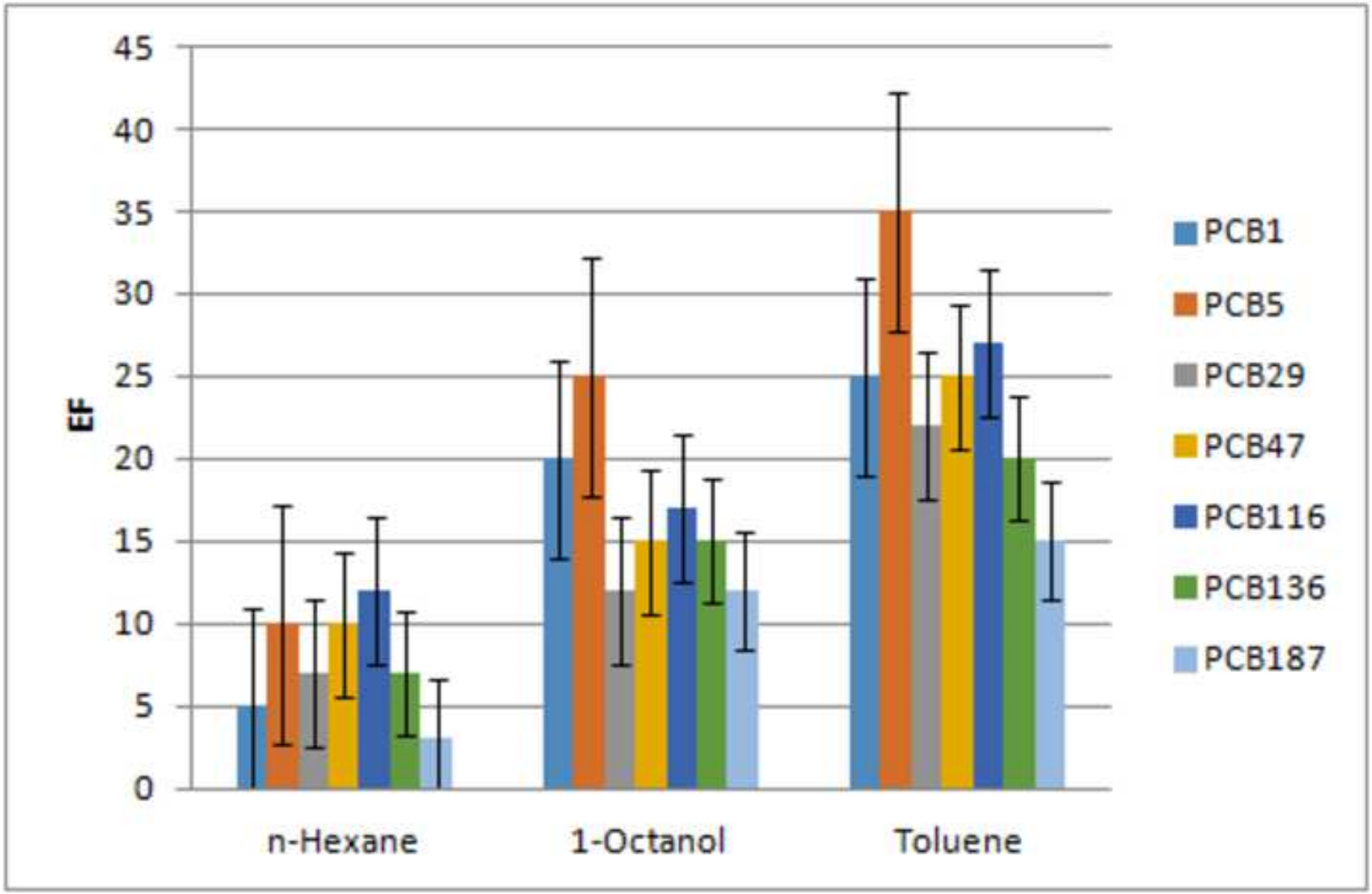


Figure 3

Click here to download high resolution image

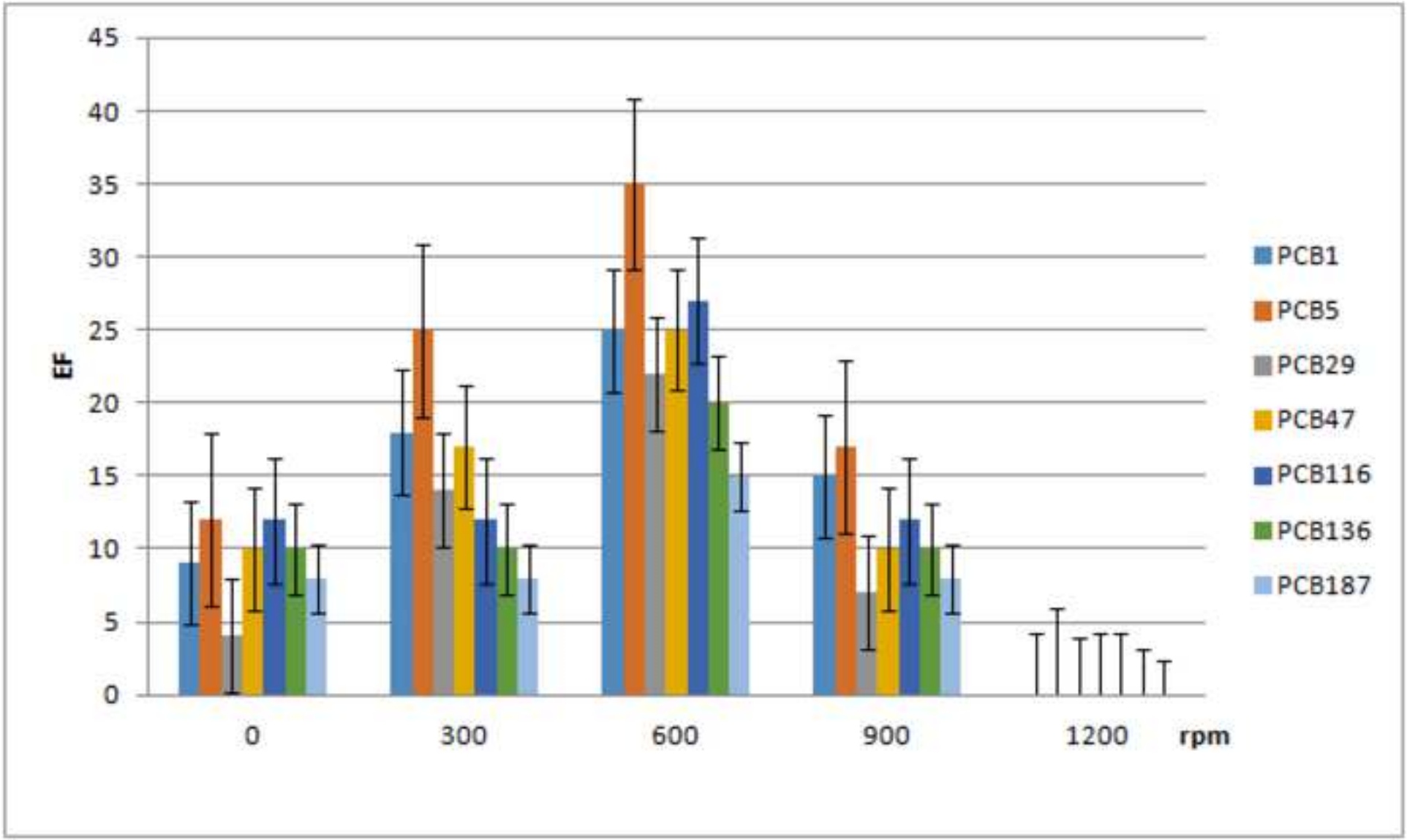


Figure 4

Click here to download high resolution image

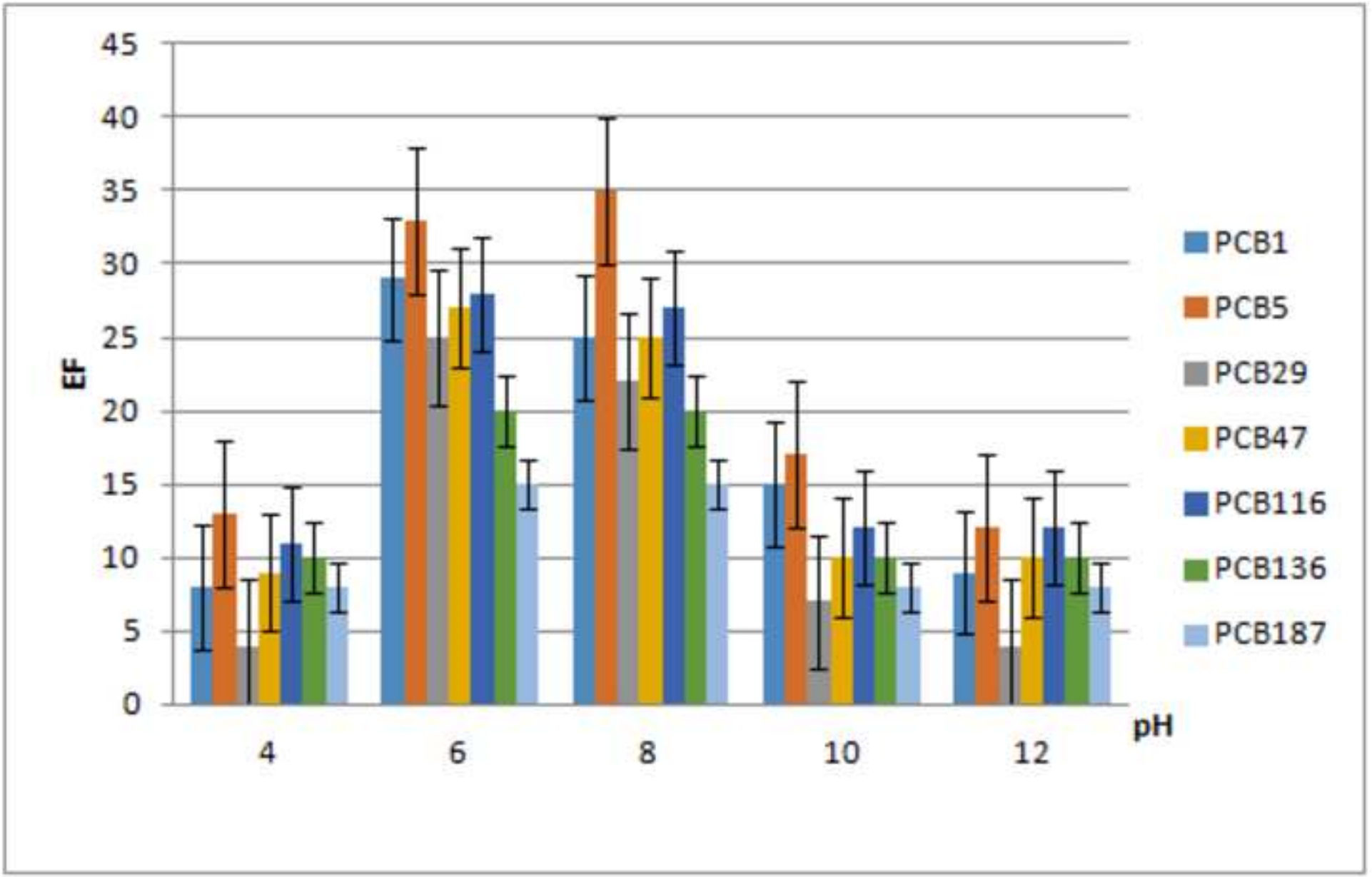


Figure 5

Click here to download high resolution image

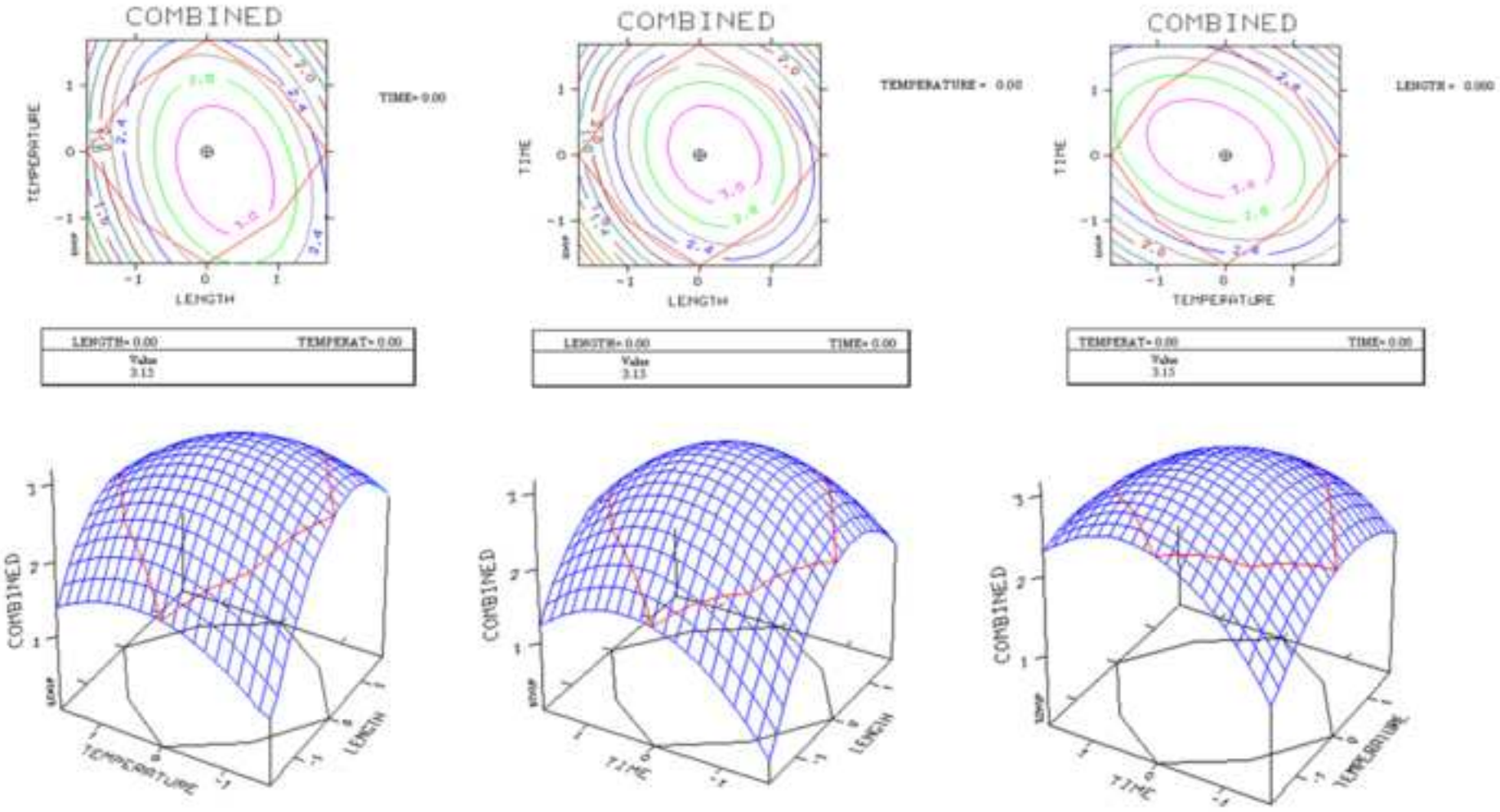
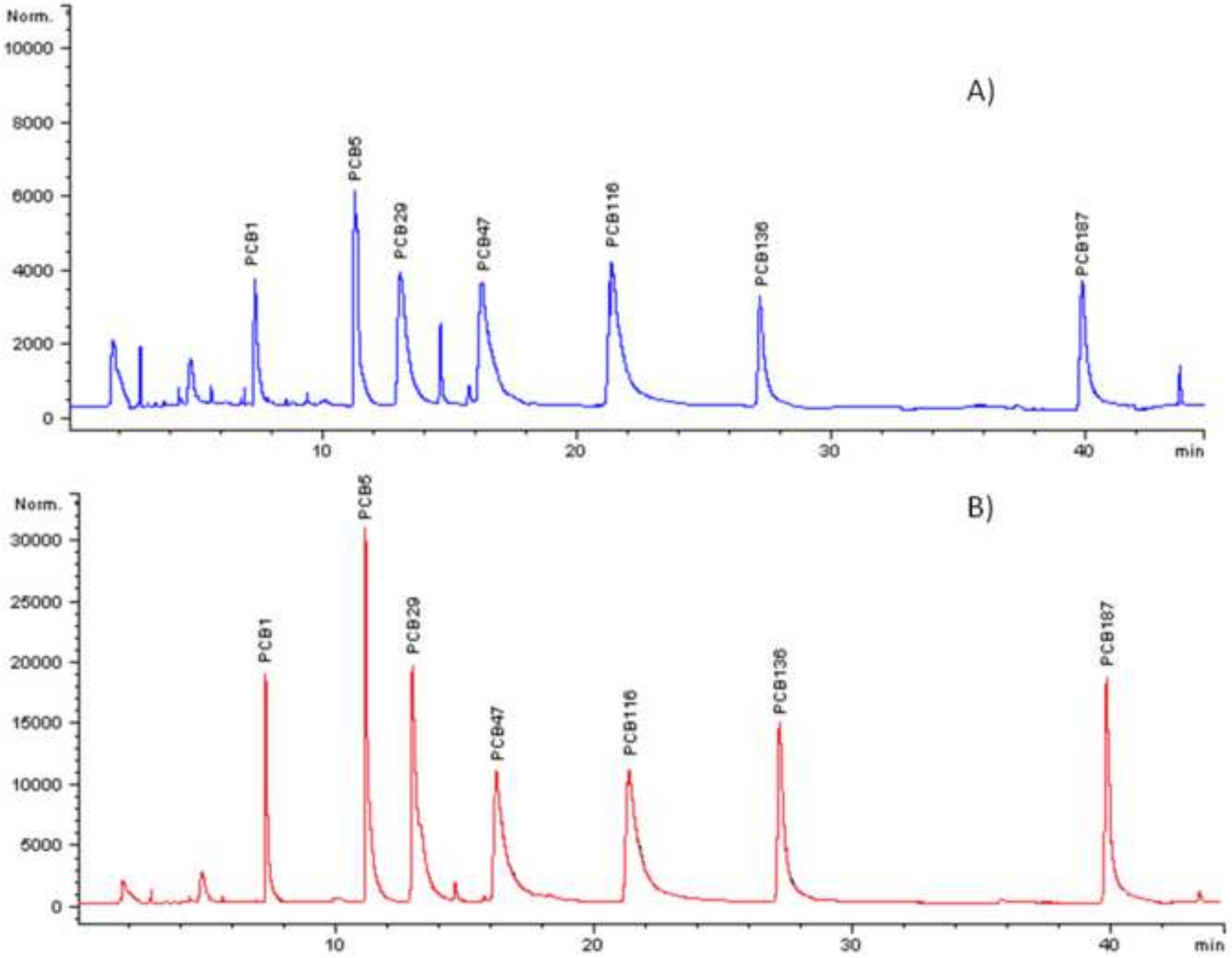


Figure 6

Click here to download high resolution image



***Credit Author Statement**

MCVA: Investigation; BCL: Investigation; GRM: Investigation; JLGA: writing-review and editing, resources; AAB: validation, visualization, methodology, investigation, supervision, Writing - original draft, review and editing; TGB: conceptualization, methodology, investigation, supervision, Writing - original draft, review and editing, funding acquisition, resources.