Journal of Chromatographic Science 2012;50:469–476
doi:10.1093/chromsci/bms030 Advance Access publication April 12, 2012

Article

A Pretreatment Method for HPLC Analysis of Cypermethrin in Microbial Degradation Systems

Shuliang Liu[1,2], Kai Yao[1]*, Dongying Jia[1], Nan Zhao[2], Wen Lai[2] and Huaiyu Yuan[2]

[1]College of Light Industry and Food, Sichuan University, 24 South Section One, Yihuan Road, Chengdu 610065, P.R. China, and [2]College of Food Science, Sichuan Agricultural University, 46 Xinkang Road, Yaan 625014, P.R. China

*Author to whom correspondence should be addressed. Email: yaokai555@yahoo.com.cn

Received 3 March 2011; revised 21 May 2011

In this paper, a pretreatment method for high-performance liquid chromatography (HPLC) determination of cypermethrin (CY) in microbial degradation systems was systemically studied, primarily to solve the problem of inaccurate determination of CY concentration caused by its uneven distribution in the systems. A suitable pretreatment method was established, including sampling, extraction and dehydration of CY. Partial sampling could be taken for bacterial and yeast systems in which CY was uniformly dispersed by an emulsifying agent, while total sampling was only suitable for mold systems with or without an emulsifying agent. CY could be fully extracted from the samples in which microbial cells were disrupted by ultrasonic treatment with acetonitrile under ultrasonic condition. The extract could be effectively dehydrated and purified by passing it through an anhydrous Na_2SO_4 column followed by an elution with acetonitrile. The determination of CY in the pretreated sample by HPLC showed a high precision [relative standard deviation (RSD) = 1.14%, $n = 5$] and a good stability over a period of five days (RSD = 1.57%, $n = 5$). The recoveries of CY in microbial degradation systems at three different spiked levels ranged from 95.68 to 108.09% (RSD = 0.50–5.87%, $n = 5$).

Introduction

Cypermethrin (CY), a synthetic pyrethroid pesticide, has been widely used in controlling pests in tea, fruits and vegetables, and in fighting against animal or human parasites (1–4). At present, it is considered as one of primary pesticide residues in agricultural products (4–6). CY can be accumulated in the human body via food, leading to some side effects on nervous, cardiovascular, reproductive and immune systems (7–10).

Biodegradation, especially microbial degradation, is generally considered to be an effective measure to eliminate pesticide residues. Since 1940, most researches have focused on the biodegradation of organophosphorus pesticides, but less on synthetic pyrethroids. Screening of microbes able to degrade pyrethroids is underway. Due to the poor water solubility of pyrethroid pesticides and the complexity of the culture systems (including medium components, microbial cells, pesticide, solvents and microbial metabolites), screening of the microbes should be performed as follows. First, dissolve pyrethroid pesticide in an organic solvent to yield a stock solution and add it quantitatively to a particular medium. Second, extract and purify residual pesticide in the culture after incubation (11). Third, determine its content by gas chromatography (GC) (12–18) or high-performance liquid chromatography (HPLC) (19, 20). Finally, calculate its degradation efficacy and determine degrading strain.

GC with a high sensitivity is widely used in the quantification of pyrethroid pesticides, but the sample must be pretreated by a complicated and strict process to obtain a higher purity. Although HPLC can analyze samples of relatively low purity, pretreatment of the samples significantly affects the accuracy of the results. Moreover, pretreatment methods of pyrethroid pesticides are not similar in different samples such as agricultural products or soil and microbial degradation systems.

Generally, microbial degradation system of pyrethroid pesticide has the following two features. The first is that pyrethroid pesticide can be added to the liquid medium with a concentration range of 50–200 mg/L (13, 14, 17, 19, 21, 22). The other is that ethanol (15, 23) or acetone (14) is usually used as a solvent, and that Tween (19, 24) acts as not only an emulsifier, but also an auxiliary solvent. Therefore, pyrethroid pesticide is often added to the medium with or without the emulsifier. In addition, some other characteristics were also found in our previous study. First, when only one kind of solvent or lower concentrations of emulsifier were used in the microbial degradation system, some of the pesticide may precipitate after incubation, resulting in its uneven dispersion in the culture. Second, microbial cells could absorb some of the pesticide during incubation. Third, mold in liquid medium could form mycelium pellets, thus causing an uneven distribution of the pesticide between the mycelia and medium in the system.

Based on the characteristics of pyrethroid pesticide degradation by microbes mentioned previously, it is necessary to establish an accurate pretreatment method for its HPLC analysis. However, little work on it has been reported so far. In this paper, a pretreatment process was investigated, including sampling, extraction and dehydration for HPLC analysis of residual CY in microbial degradation system. The primary purpose of this study is to provide a method for accurately determining pyrethroid pesticide in microbial degradation systems by HPLC.

Experimental

Chemicals and reagents

Analytical standard of CY (99.7%) was purchased from Chinese Certified Reference Materials/Reference Material (CRM/RM) Center (Beijing, China). CY pesticide (96.8%) was from Rongcheng Chemical Reagent Company (Nanjing, China). Acetonitrile of HPLC grade was obtained from CNW Technologies GmbH (Dusseldorf, Germany). Petroleum ether, *n*-hexane, chloroform, anhydrous Na_2SO_4 and anhydrous $MgSO_4$ were purchased from Kelong Chemical Agents Company (Chengdu, China).

Ultrapure water obtained from a Milli-Q water purification system (Bedford, MA) was used in mobile phases, which were filtered through a 0.45 μm cellulose acetate (water) or polytetrafluoroethylene (PTFE) (acetonitrile) filter and degassed via ultrasound before use.

All other chemicals and reagents were of analytical grade and purchased from commercial sources, unless otherwise stated.

Preparation of stock and working solutions

Stock solutions of CY standard and CY pesticide (1.0 mg/mL) were exactly prepared with acetonitrile, respectively, and stored in lightproof containers at 4°C. Then, each was diluted with acetonitrile to give the final concentrations of 0.053, 0.105, 0.525, 1.050, 5.250, 7.875, 10.500 and 31.500 μg/mL, respectively, for the calibration curve.

Two kinds of working solutions with 10.0 mg/mL of CY pesticide were prepared as follows. Working Solution I was obtained by directly dissolving CY pesticide with ethanol. CY pesticide was first mixed with Tween 80 in a volume ratio of 1:2 and then adjusted with ultrapure water to reach the desire volume, thus resulting in Working Solution II. Both working solutions were stored at 4°C.

Microorganism isolation and incubation conditions

Bacillus licheniformis B-1 (B-1) was isolated from the soil of a tea garden, *Lactobacillus pentosus* C50-6 (C50-6) from a traditional Chinese sausage, *Saccharomyces cerevisiae* H-2 (H-2) from distillers' grains and *Aspergillus oryzae* M-4 (M-4) from a soy sauce koji. After being gradually domesticated with CY pesticide, the four strains could tolerate 750 mg/L of CY.

The components of four different media were as follows: (i) Mineral Salts Medium (MS) for H-2 consisted of 0.15% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.15% K_2HPO_4, 0.02% $MgSO_4^-7H_2O$ and 0.05% NaCl (pH 7.5); (ii) De Man, Rogosa and Sharpe Medium (MRS) for C50-6 was constituted of 1.0% beef extract, 1.0% tryptone, 0.5% yeast extract, 2.0% glucose, 0.5% sodium acetate, 0.2% diammonium hydrogen citrate, 0.1% Tween 80, 0.058% $MgSO_4^-7H_2O$ and 0.028% $MnSO_4-4H_2O$ (pH 6.2-6.4); (iii) Potato Dextrose Medium (PD) for M-4 was composed of 20% potato and 2.0% glucose; (iv) Luria–Bertani Medium (LB) for B-1 was composed of 1.0% peptone, 0.5% yeast extract and 1.0% NaCl (pH 7.0). Their corresponding solid media were obtained by adding 1.8% agar to the previously described liquid media. After the media (liquid and solid) were autoclaved at 121°C for 15 min, CY pesticide working solutions were then added to them to make a CY concentration of 100 mg/L.

The suspension, 1.5 mL (1.0×10^8 cfu/mL) of B-1, C50-6, M-4 or H-2 was inoculated into a 250-mL Erlenmeyer flask with 30 mL of its corresponding liquid medium, respectively. In the blank control, the strain suspension was replaced by the sterile water. All the blank and test media were incubated at 180 rpm and 30°C for 120 h in a shaker, and then the blank cultures and test cultures were obtained.

HPLC conditions and analysis

The concentrations of CY standard, CY pesticide and the residual CY pesticide in microbial degradation systems were analyzed on a Shimadzu HPLC (LC-10A2010C HT, Japan) equipped with UV detector, column oven, electric sample valve, Phenomenex Gemini C18 reversed-phase column (5.0 μm, 150 × 4.60 mm, i.d.) and C18 guard column (4 × 2 mm, i.d.) (Torrance, CA). The data were processed by Shimadzu LC-solution software.

Based on the reports (23, 25–27) and pre-test results obtained in our lab, CY determination was performed using a mobile phase of acetonitrile–ultrapure water (85:15, *v*/*v*) at a flow rate of 1.0 mL/min. Ten microliters of the sample was injected, and CY was detected at 210 nm under 25°C. Under the previously described conditions, the retention time (RT) for CY was approximately 7.0–7.5 min.

Pre-treatment method

Selection of solvents

Four aliquots of 5.0 mL ethanol–CY MS medium containing 100 mg/L of CY pesticide were mixed with 10 mL of *n*-hexane-acetone (39:1, *v*/*v*), petroleum ether-acetone (39:1, *v*/*v*), chloroform and acetonitrile, respectively, and were shaken on an oscillator for 60 s, thus resulting in four extracts. Then, 2.0 g of NaCl was added to acetonitrile extract to delaminate. All the extracts were kept at room temperature for 10 min, and the organic phase obtained was centrifuged at 14,000 × g for 10 min in a microcentrifuge (Sorvall Pico, Thermo). Each supernatant was then diluted five times with its corresponding extraction solvent, and its CY concentration was determined by HPLC under the conditions described previously. Extraction efficacy was calculated with the following equation: Extraction efficacy (%) = (CY concentration in the extract / 100.0) × 100.

Selection of dehydration methods

Ethanol-CY MS medium (1.0 mL) containing 100 mg/L of CY pesticide was mixed with an equal volume of acetonitrile, and water in the mixture was removed by the following three methods, respectively. In the first method, the mixture was thoroughly mixed with 0.4 g NaCl and then centrifuged at 14,000 × g for 30 s to facilitate its delamination. The resulting mixture was fully mixed with 0.8 g anhydrous $MgSO_4$ and kept at room temperature for 10 min for its dehydration. The obtained supernatant (0.1 mL) was diluted 10 times with acetonitrile and centrifuged at 14,000 × g for 10 min. After that, the supernatant was filtered through a PTFE Millipore membrane (0.45 μm). Finally, 10 μL of the filtrate was then injected into the HPLC column, and the CY concentration in it was detected. In the second method, the same steps described in the first method were performed, except that anhydrous $MgSO_4$ was replaced by anhydrous Na_2SO_4. In the third method, a glass column (80 × 15 mm, i.d.) was first filled with anhydrous Na_2SO_4 and pre-balanced with acetonitrile. The previously described mixture (2.0 mL) was loaded on the column and eluted with acetonitrile (approximately 8 mL). The final volume of the elution was adjusted to 10.0 mL with acetonitrile. Then, centrifugation, filtration and injection into the HPLC column were done as described in the first method. An optimum method was judged by CY extraction efficacy.

Selection of sampling methods

Working Solution I or II was added to MS, MRS, PD and LB media, respectively, to obtain ethanol-CY media or Tween 80-CY media containing 0.2% Tween 80. Based on the higher solubility of CY in ethanol than in water and the emulsification of Tween 80, four ethanol-Tween 80-CY media were prepared by adding Working Solution I to MS, MRS, PD and LB media containing 0.2% Tween 80. All of the three types of media contained 100 mg/L of CY pesticide. Then, each of the four strains was inoculated into its corresponding medium as described previously. The test cultures and their corresponding blanks for each strain were totally and partially sampled. Total sampling was performed by taking the whole volume of the cultures, but partial sampling was done by taking 1.0 or 2.0 mL out of the cultures. CY pesticide in the samples was then extracted with acetonitrile and dehydrated using the method established previously. The concentrations of CY in the extracts were then determined by HPLC.

Selection of ultrasonic-assisted extraction

The culture sample was first disrupted at 40 kHz and 300 W for 30 min in ultrasonic equipment (AS 100200A, Tianjin, China). Then it was mixed with equal volume of acetonitrile and re-ultrasonized (40 kHz, 300 W) for 15 min to extract residual CY pesticide. Two milliliters of the extract was dehydrated according to the method established previously and analyzed by HPLC. The sample without ultrasonic treatment was a blank control. It was determined whether ultrasonic should be applied or not by comparing the content of residual CY in the two kinds of samples.

Meanwhile, wet microbial cells were harvested by centrifuging whole culture in a bottle at 2,000 × g for 10 min; following this, the cells were weighed. After that, a 3-times volume of acetonitrile–water (1:1, *v*/*v*), as much as the mass of the cells, was added to the cells and mixed thoroughly. The resulting cell suspension was ultrasonized in an ice bath at 20 kHz and 475 W for 10 min with 2 s of work and 1 s of a break using an ultrasonic cell disruptor (Scientz-IID, Ningbo, China) and was centrifuged (12,500 × g) at 4°C for 20 min. Two milliliters of the supernatant was dehydrated by the method established previously, and its CY concentration was determined by HPLC. The necessity of ultrasonic-assisted extraction was further evaluated according to CY content in the cells.

Validation of procedure

The precision and accuracy of HPLC analysis on residual CY pesticide in microbial degradation systems were studied. The culture with residual CY pesticide from M-4 degradation system containing 100 mg/L of CY was used for precision determination. Ten microliters of the sample, pretreated according to the method established previously, was injected into HPLC column, which was repeated 5 times. Moreover, for stability test of the sample, the pretreated sample was kept at room temperature (25°C) for 120 h, and each sampling was done at an interval of 24 h. For recovery determination, CY standard stock solution (1.0 mg/mL) was added to the cultures of the four strains at concentrations of 10.0, 50.0 and 100.0 mg/L, respectively. Then, the culture with CY standard stock solution was kept at 25°C for 30 min before extraction to ensure that the CY standard was thoroughly dispersed.

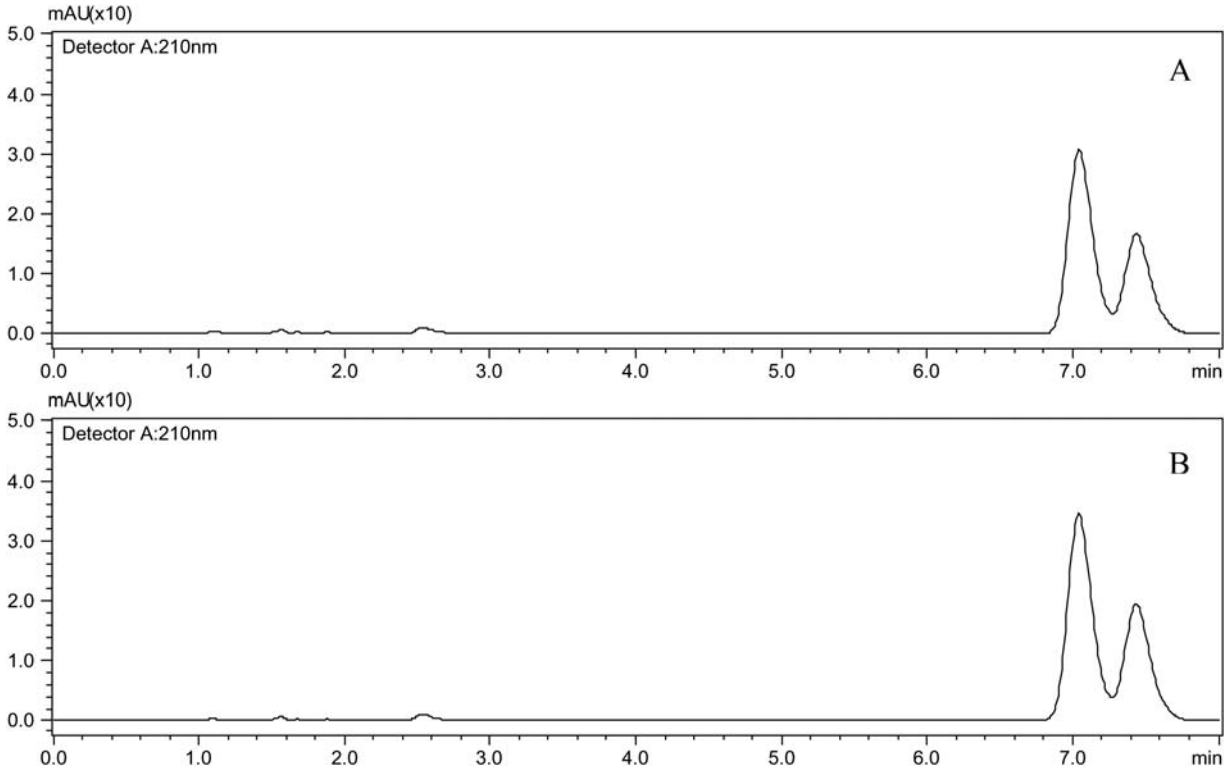

Figure 1. HPLC chromatograms of CY standard (A) and CY pesticide (B).

Results and Discussion

Calibration study

Figure 1 shows HPLC chromatograms of CY standard and CY pesticide. In the figure, both have two peaks with the same retention time, which appeared at 7.040 and 7.434 min, respectively. The sum of the two peak areas was used for calculation. The calibration curve of CY standard obtained under the given chromatographic conditions showed a good linearity relationship at CY concentrations ranging from 0.053 to 31.500 μg/mL ($R^2 = 1.0000$). The linear range of concentrations encompassed the estimated concentrations of CY pesticide added or residual in cultures.

Pretreatment method

The microbial degradation system of CY is very complex. It contains medium components, microbial cells, CY and solvents (ethanol and Tween 80), as well as microbial metabolites. Therefore, it is necessary to set up a pretreatment method for the accurate quantitative analysis of CY in microbial degradation systems by HPLC.

Suitable solvent for CY extraction

MS medium, a basic medium, is representative of most microbial degradation systems of CY. In addition, ethanol is the best solvent of CY in the system. Moreover, a preliminary experiment showed that the distribution of CY in MS medium with Working Solution I was uniform before shake incubation. Therefore, any samples from the ethanol–CY MS media can be applied for CY extraction. Among the four solvents used, acetonitrile had the highest extraction efficiency (55.84%), followed by chloroform (25.67%), *n*-hexane-acetone (22.83%) and petroleum ether–acetone (17.51%). Because ethanol present in liquid–liquid extraction system has a strong affinity with CY, it

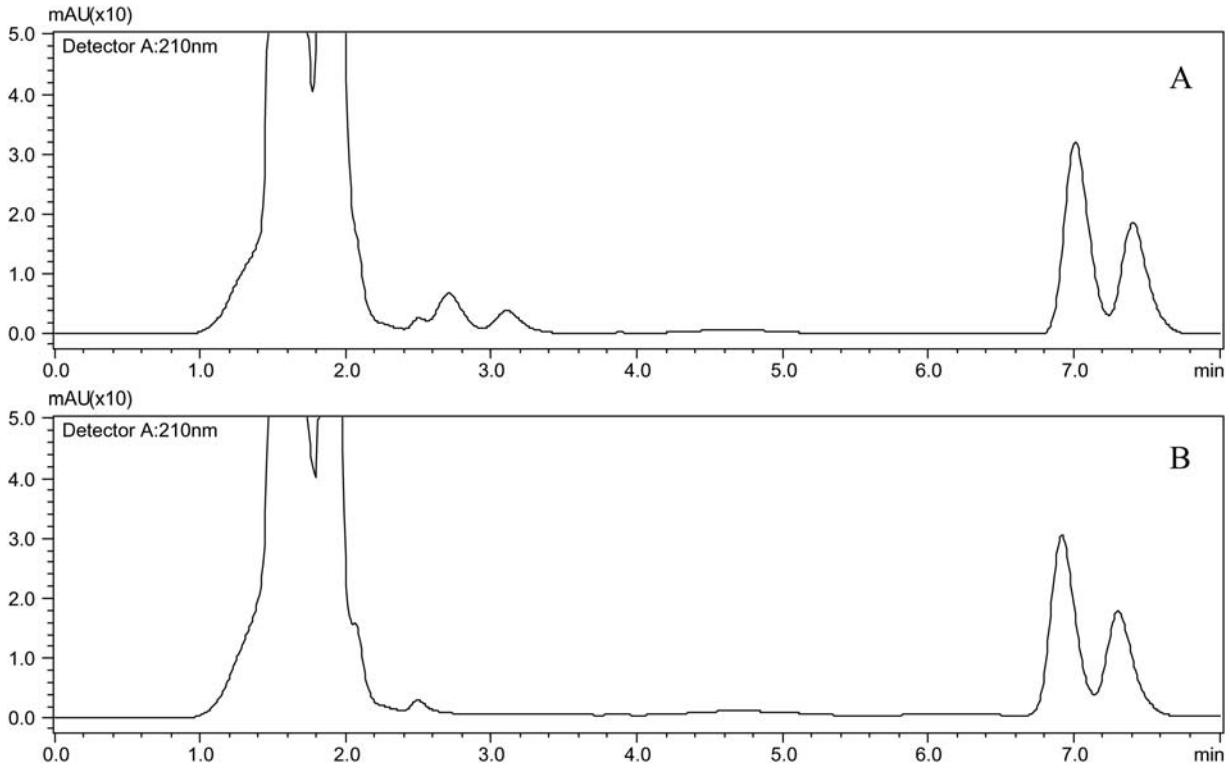

Figure 2. HPLC chromatograms of CY extract before (A) and after (B) passing anhydrous Na_2SO_4 column.

Table I
Effect of Sampling Methods on CY Extraction from Four Blank Cultures

Blank culture	CY concentration (mean ± SD, mg/L) (RSD, %)* ($n = 5$)					
	Partial sampling			Total sampling		
	Ethanol–CY	Tween 80–CY	Ethanol–Tween 80–CY	Ethanol–CY	Tween 80–CY	Ethanol–Tween 80–CY
MS	51.48 ± 27.97 (54.33)	98.50 ± 0.73 (0.74)	107.65 ± 3.33 (3.09)	102.53 ± 1.21 (1.18)	99.57 ± 1.53 (1.54)	100.53 ± 2.20 (2.19)
PD	68.75 ± 31.22 (45.41)	98.75 ± 2.38 (2.41)	99.86 ± 2.34 (2.34)	100.13 ± 1.06 (1.06)	98.43 ± 1.25 (1.27)	101.18 ± 0.28 (0.28)
LB	78.05 ± 16.88 (21.63)	102.44 ± 3.53 (3.45)	100.21 ± 3.84 (3.83)	101.41 ± 2.05 (2.02)	99.18 ± 2.99 (3.01)	101.43 ± 2.83 (2.79)
MRS	86.05 ± 11.57 (13.44)	98.44 ± 3.07 (3.12)	97.88 ± 2.33 (2.38)	99.01 ± 1.72 (1.74)	101.21 ± 1.39 (1.37)	98.46 ± 2.49 (2.53)

*RSD = 100% × (SD/mean).

can increase solubility of CY in aqueous phase. Compared with the other three solvents with lower polarity, acetonitrile shows much better compatibility with ethanol, and thus it can extract more CY from the aqueous phase. Therefore, acetonitrile was chosen to be a suitable solvent for extracting CY from media or cultures.

Suitable method for sample dehydration

The addition of NaCl to CY acetonitrile extract from ethanol–CY MSM was favorable for the formation of aqueous phase. However, some of CY still remained in it, thus resulting in lower extraction efficiency. Therefore, dehydration treatment for the extract should be considered. Anhydrous Na_2SO_4 and anhydrous $MgSO_4$ are water-absorbents commonly used to remove water in organic phases. When they were directly added to acetonitrile extract, the extraction efficacies of CY were 88.20 and 87.91% ($n = 5$), respectively. To further improve extraction efficiency, a column filled with water-absorbent was applied. However, anhydrous $MgSO_4$ could release heat and coagulate during water absorption, thus obstructing CY elution. Therefore, anhydrous Na_2SO_4 was an ideal filler of the dehydration column. When acetonitrile extract from MS medium was loaded onto anhydrous Na_2SO_4 column and eluted with acetonitrile, extraction efficacy of CY from the medium could be increased to 98.54% ($n = 5$). As shown in Figure 2, some impurity peaks in the extract disappeared after it was passed through anhydrous Na_2SO_4 column. This indicates that the adoption of anhydrous Na_2SO_4 column can not only significantly improve the extraction efficacy of CY, but also remove some water-soluble impurities. The impurities had little influence on the determination for CY, but removing the impurities as much as possible could prolong the life of the chromatographic column.

Table II
Effect of Sampling Methods on CY Extraction from Ethanol–Tween 80–CY Medium

Strain	CY concentration (mean ± SD, mg/L) (RSD, %)* (n = 5)	
	Partial sampling	Total sampling
H-2	91.42 ± 1.10 (1.20)	91.46 ± 1.84 (2.01)
M-4	36.19 ± 1.53 (4.24)	56.78 ± 0.05 (0.08)
B-1	41.23 ± 0.89 (2.17)	42.09 ± 0.83 (1.97)
C50-6	88.36 ± 0.90 (1.02)	88.64 ± 1.01 (1.14)

*RSD = 100% × (SD / mean).

Table III
Effect of Ultrasonic on CY Extraction from Ethanol–Tween80–CY Medium

Strain	CY concentration (mean ± SD, mg/L) (RSD, %)* (n = 5)		
	Acetonitrile extraction	Acetonitrile extraction assisted with ultrasonic	Microbial cells of 1-L culture
H-2	91.42 ± 1.10 (1.20)	100.31 ± 1.48 (1.48)	8.81 ± 0.20 (2.31)
M-4	57.28 ± 1.00 (1.74)	73.65 ± 0.52 (0.71)	17.63 ± 0.24 (1.34)
B-1	42.09 ± 0.59 (1.39)	46.23 ± 0.72 (1.56)	5.56 ± 0.10 (1.74)
C50-6	88.36 ± 0.90 (1.02)	99.95 ± 2.10 (2.10)	10.64 ± 0.23 (2.17)

*RSD = 100% × (SD / mean).

Confirmation of sampling method

Blank cultures of each strain in the three types of media were partially and totally sampled and pretreated as the method established. Their CY concentrations were then measured by HPLC. The results are shown in Table I. In the partial sampling of the four blank cultures from ethanol–CY media, CY concentration was much lower than 100 mg/L, and their relative standard deviations (RSDs) varied from 13.44 to 54.33%, indicating poor reproducibility. This demonstrates that CY is unevenly dispersed in these cultures when ethanol is used as the sole solvent. Moreover, a yellow oily substance was observed in the cultures after shaking. However, for those blank cultures from Tween 80–CY media or ethanol–Tween 80–CY media, all the extraction efficiencies were approximately 100% and their RSDs changed little, only from 0.74 to 3.45% or from 2.34 to 3.83%, respectively, showing good reproducibility. Therefore, it can be assumed that partial sampling may be suitable for the cultures containing Tween 80, which can ensure that CY is uniformly dispersed in the cultures. While in total sampling, the RSDs of all the blank cultures with the tested concentration at approximately 100 mg/L were in the range of 0.28 to 3.01%.

Based on the results from blank cultures, a sampling method for microbial cultures was further investigated, in which only ethanol–Tween 80–CY medium was used for incubating the four strains. This design was proposed based on the following three reasons: (i) little difference was observed between the

Table IV
Recoveries of CY in Four Microbial Degradation Systems ($n = 5$)

Medium + strain	CY concentration (mean ± SD, mg/L)	Spiked concentration (mg/L)	Measured CY concentration (mean ± SD, mg/L)	Recovery concentration (mean ± SD, mg/L)	Recovery (%)	RSD* (%)
MS + H-2	98.70 ± 3.20	100	197.52 ± 3.87	98.82 ± 0.74	98.82	0.75
		50	147.20 ± 3.35	48.50 ± 0.35	97.00	0.73
		10	109.24 ± 3.29	10.53 ± 0.31	105.30	2.90
PD + M-4	74.40 ± 1.81	100	182.49 ± 1.91	108.09 ± 0.54	108.09	0.50
		50	127.45 ± 2.65	53.05 ± 1.76	106.10	3.32
		10	84.42 ± 1.61	10.02 ± 0.33	100.16	3.34
LB + B-1	45.12 ± 1.02	100	148.72 ± 3.74	103.60 ± 3.46	103.60	3.34
		50	93.45 ± 1.70	48.33 ± 1.26	96.66	2.62
		10	55.69 ± 1.20	10.56 ± 0.62	105.57	5.87
MRS + C50-6	102.60 ± 2.06	100	202.79 ± 1.59	100.19 ± 1.85	100.19	1.85
		50	152.81 ± 2.53	50.21 ± 1.09	100.41	2.16
		10	112.16 ± 2.13	9.57 ± 0.09	95.68	0.93

*RSD = 100% × (SD / mean).

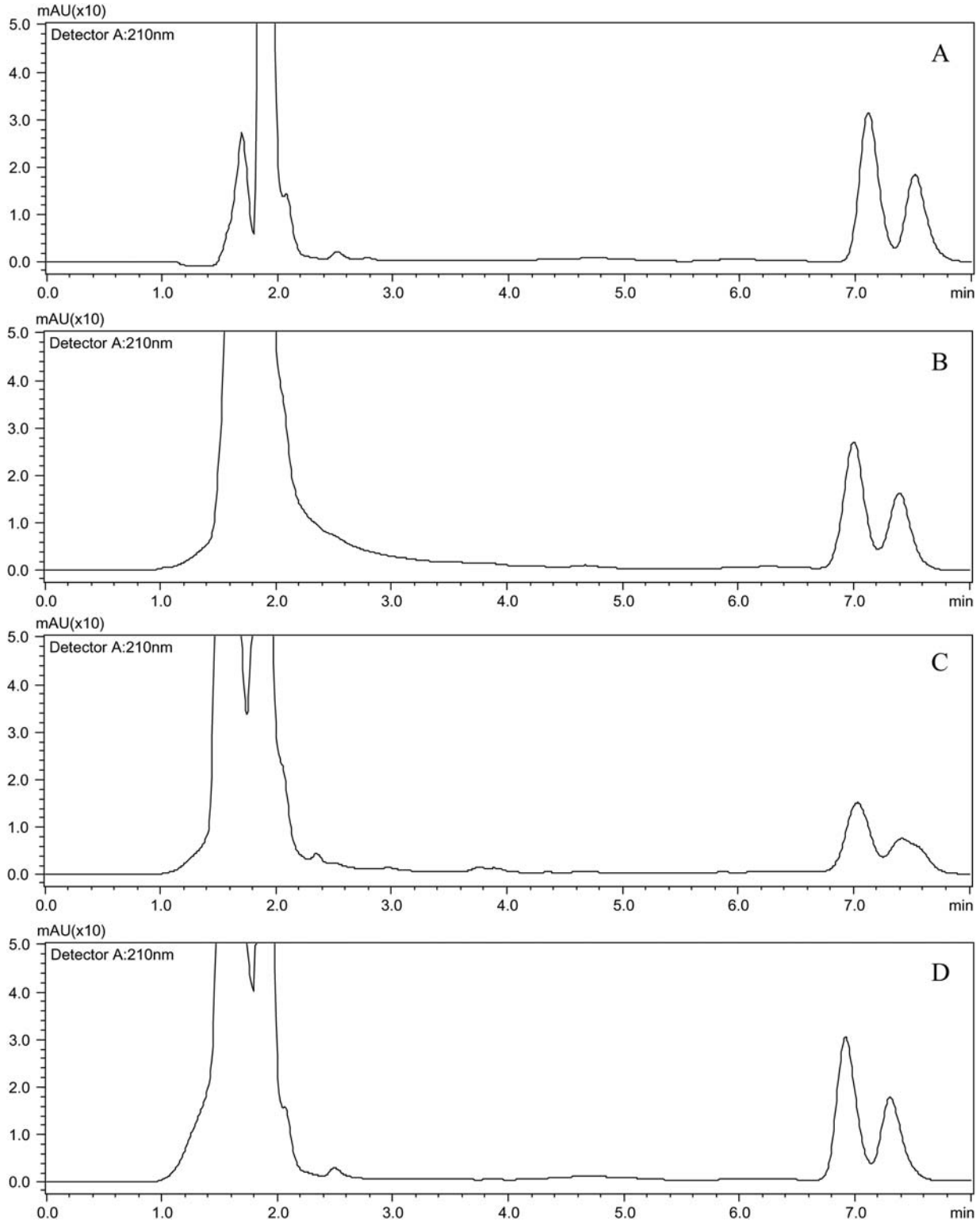

Figure 3. HPLC chromatograms of CY in four microbial degradation systems. MS + H-2 (A), PD + M-4 (B), LB + B-1 (C), MRS + C50-6 (D).

extraction efficiencies of CY from ethanol–Tween 80–CY medium and Tween 80–CY medium in either sampling manner, and a similar result was obtained between ethanol–Tween 80–CY medium and ethanol–CY medium in total sampling manner; (ii) ethanol–Tween 80–CY medium exerts an advantage over the other two types of media, that is, better solubility of CY in ethanol and well-emulsified state of CY in Tween 80; (iii) CY is soluble in ethanol but insoluble in water,

so it is easy to prepare Working Solution I but difficult to prepare Working Solution II (as described previously).

Table II demonstrates the effects of sampling methods on CY extraction from ethanol–Tween 80–CY medium. As shown in Table II, CY was uniformly dispersed in the cultures of B-1, C50-6 and H-2 containing Tween 80, for either culture of the three strains, and CY concentrations in the cultures from partial and total sampling were found to be almost identical. However, for M-4 culture, the two sampling methods led to a significant difference in residual CY concentrations in the samples (36.19 mg/L and 56.78 mg/L, respectively). This is due to the formation of large mycelial pellets during shaking, which may absorb some of CY pesticide, thus causing the uneven distribution of CY in the culture.

In the following studies, partial sampling was applied in bacterial and yeast degradation systems containing Tween-80, and total sampling used only in the mold system with Tween-80 based on the preceding results.

Necessary of ultrasonic-assisted extraction

The results of acetonitrile extractions with and without ultrasonic are shown in Table III. Because ultrasonic can disrupt microbial cells, the former method had a higher extraction efficacy than the latter one for each strain. Thus, it was validated that microbial cells absorbed some CY, but the abilities to do so were different depending on their species. Among them, M-4 had the highest absorption value on CY (17.63 mg/L culture). Therefore, ultrasonic-assisted extraction of CY in all the cultures is necessary. This explains why CY concentration in the culture was rather lower after microbial cells were centrifugally removed.

Most researchers have used organic solvents with low polarity to extract CY from microbial degradation systems (17, 19). However, when the solvents (*n*–hexane–acetone, petroleum ether–acetone or chloroform) with polarity similar to that of CY was used as an extractant and when a suitable amount of emulsifier was added to the medium, two obvious problems appeared. One was that the extract had difficulty formimg an organic layer quickly due to a stronger emulsifying effect. The other was that CY that accumulated in microbial cells could not be fully extracted using only one of the solvents. Therefore, the pretreatment method was improved by disrupting microbial cells with ultrasonic treatment and then extracting CY with acetonitrile under ultrasonic to avoid the emulsification and to effectively extract CY from the microbial cells.

Validation procedure

Precision and stability studies

The results from the precision study showed that the RSD of CY concentrations in the *A. oryzae* degradation system was only 1.14%. Stability study demonstrated that the RSD of CY concentrations in the samples left at room temperature for different times was 1.57%. These results suggest that when a sample from microbial degradation system is pretreated by the established method, the HPLC determination of CY concentration in it has high precision and good stability.

Recovery of standard addition

CY concentrations in spiked samples were detected by HPLC and the recoveries are shown in Table IV. The recoveries of CY in all microbial degradation systems changed from 95.68 to 108.09% and their RSDs varied from 0.50 to 5.87%. Moreover, no interference peak was observed in all HPLC chromatograms, as shown in Figure 3.

Conclusions

In this study, a suitable pretreatment method has been established that includes sampling, extraction and dehydration. As for bacterial and yeast cultures in which CY is uniformly dispersed by an emulsifying agent, partial sampling can be adopted. As for mold culture with or without an emulsifying agent, total sampling is only one choice for small-scale experimental study, but it is not feasible for applications in large-scale microbial degradation systems of CY. Thus, it is needed to perform a further study on its sampling method. After the microbial cells in the culture are disrupted by ultrasonic treatment, the CY can be fully extracted with acetonitrile under ultrasonic condition. The resulting extract is loaded onto an anhydrous Na_2SO_4 column and eluted with acetonitrile to remove water and some water-soluble impurities. This pretreatment method can make HPLC determination of CY in microbial degradation systems have high precision and good stability as well as high recovery.

Acknowledgments

The authors are grateful to the National Natural Science Foundation of China (21072137) for financial support of this research.

References

1. Arthur, F.H., Campbell, J.F.; Distribution and efficacy of pyrethrin aerosol to control *Tribolium confusum* (Coleoptera: Tenebrionidae) in food storage facilities; *Journal of Stored Products Research*, (2008); 44: 58–64.
2. Starr, J., Graham, S., Stout, D., II, Andrews, K., Nishioka, M.; Pyrethroid pesticides and their metabolites in vacuum cleaner dust collected from homes and day-care centers; *Environmental Research*, (2008); 108: 271–279.
3. Maltby, L., Hills, L.; Spray drift of pesticides and stream macroinvertebrates: Experimental evidence of impacts and effectiveness of mitigation measures; *Environmental Pollution*, (2008); 156: 1112–1120.
4. Bishnu, A., Chakrabarti, K., Chakraborty, A., Saha, T.; Pesticide residue level in tea ecosystems of Hill and Dooars regions of West Bengal, India; *Environmental Monitoring and Assessment*, (2009); 149: 457–464.
5. Chu, S., Henny, C.J., Kaiser, J.L., Drouillard, K.G., Haffner, G.D., Letcher, R.J.; Dacthal and chlorophenoxy herbicides and chlorothalonil fungicide in eggs of osprey (*Pandion haliaetus*) from the Duwamishe-Lake Washington-Puget Sound area of Washington state, USA; *Environmental Pollution*, (2007); 145: 374–381.
6. González-Rodríguez, R.M., Rial-Otero, R., Cancho-Grande, B., Simal-Gándara, J.; Occurrence of fungicide and insecticide residues in trade samples of leafy vegetables; *Food Chemistry*, (2008); 107: 1342–1347.

7. Wolansky, M.J., Harrill, J.A.; Neurobehavioral toxicology of pyrethroid insecticides in adult animals: A critical review; *Neurotoxicology and Teratology*, (2008); 30: 55–78.
8. Kolaczinski, J.H., Curtis, C.F.; Chronic illness as a result of low-level exposure to synthetic pyrethroid insecticides: A review of the debate; *Food and Chemical Toxicology*, (2004); 42: 697–706.
9. Zhang, J., Zhu, W., Zheng, Y., Yang, J., Zhu, X.; The antiandrogenic activity of pyrethroid pesticides cyfluthrin and β-cyfluthrin. *Reproductive Toxicology*, (2008); 25: 491–496.
10. Ortiz-Pérez, M.D., Torres-Dosal, A., Batres, L.E., López-Guzmán, O.D., Grimaldo, M., Carranza, C., *et al.*; Environmental health assessment of deltamethrin in a malarious area of Mexico: Environmental persistence, toxicokinetics, and genotoxicity in exposed children; *Environmental Health Perspectives*, (2005); 113: 782–786.
11. González-Rodríguez, R.M., Rial-Otero, R., Cancho-Grande, B., Simal-Gándara, J.; Determination of 23 pesticide residues in leafy vegetables using gas chromatography-ion trap mass spectrometry and analyte protectants; *Journal of Chromatography A*, (2008); 1196–1197: 100–109.
12. Grant, R.J., Betts, W.B.; Biodegradation of the synthetic pyrethroid cypermethrin in used sheep dip; *Letters in Applied Microbiology*, (2003); 36: 173–176.
13. Saikia, N., Gopal, M.; Biodegradation of β-cyfluthrin by fungi; *Journal of Agricultural and Food Chemistry*, (2004); 52: 1220–1223.
14. Saikia, N., Das, S.K.D., Patel, B.K.C., Niwas, R., Singh, A., Gopal, M.; Biodegradation of beta-cyfluthrin by *Pseudomonas stutzeri* strain S1; *Biodegradation*, (2005); 16: 581–589.
15. Liang, W.Q., Wang, Z.Y., Li, H., Wu, P.C., Hu, J.M., Luo, N., *et al.*; Purification and characterization of a novel pyrethroid hydrolase from *Aspergillus niger* ZD11; *Journal of Agricultural and Food Chemistry*, (2005); 53: 7415–7420.
16. Paingankar, M., Jain, M., Deobagkar, D.; Biodegradation of allethrin, a pyrethroid insecticide, by an *Acidomonas sp.*; *Biotechnology Letters*, (2005); 27: 1909–1913.
17. Guo, P., Wang, B., Hang, B., Li, L., Ali, S.W., He, J., *et al.*; Pyrethroid-degrading *Sphingobium sp.* JZ-2 and the purification and characterization of a novel pyrethroid hydrolase; *International Biodeterioration and Biodegradation*, (2009); 63: 1107–1112.
18. Misra, A.K., Vinod, R.S., Bhatttacharyya, A.; Degradation of fenvalerate (pyrethroid) pesticide in milk by lactic acid bacteria; *Indian Journal of Dairy Science*, (1996); 49: 635–639.
19. Maloney, S.E., Maule, A., Smith, A.R.W.; Microbial transformation of the pyrethroid insecticides: Permethrin, deltamethrin, fastac, fenvalerate, and fluvalinate; *Applied and Environmental Microbiology*, (1988); 54: 2874–2876.
20. Tallur, P.N., Megadi, V.B., Ninnekar, H.Z.; Biodegradation of cypermethrin by *Micrococcus sp.* strain CPN 1; *Biodegradation*, (2008); 19: 77–82.
21. Grant, R.J., Daniell, T.J., Betts, W.B.; Isolation and identification of synthetic pyrethroid-degrading bacteria; *Journal of Applied Microbiology*, (2002); 92: 534–540.
22. Grant, R.J., Betts, W.B.; Mineral and carbon usage of two synthetic pyrethroid degrading bacterial isolates; *Journal of Applied Microbiology*, (2004); 97: 656–662.
23. Enayati, A.A., Vontas, J.G., Small, G.J., Mccarroll, L., Hemingway, J.; Quantification of pyrethroid insecticides from treated bednets using a mosquito recombinant glutathione S-transferase; *Medical and Veterinary Entomology*, (2001); 15: 58–63.
24. Liao, M., Zhang, H.J., Xie, X.M.; Isolation, identification and degradation characteristics of a *Pseudomonas diminuta* strain able to degrade pyrethriod pesticides; *Acta Scientiae Circumstantiae*, (2009); 29: 1388–1394.
25. Martel, A.C., Zeggane, S.; Determination of acaricides in honey by high-performance liquid chromatography with photodiode array detection; *Journal of Chromatography A*, (2002); 954: 173–180.
26. Cheng, J.H., Liu, M., Yu, Y., Wang, X.P., Zhang, H.Q., Ding, L., *et al.*; Determination of pyrethroids in porcine tissues by matrix solid-phase dispersion extraction and high-performance liquid chromatography; *Meat Science*, (2009); 82: 407–412.
27. Galera, M.M., Garcca, M.D.G., Valverde, R.S.; Determination of nine pyrethroid insecticides by high-performance liquid chromatography with post-column photoderivatization and detection based on acetonitrile chemiluminescence; *Journal of Chromatography A*, (2006); 1113: 191–197.