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dinotefuran concentration in Japanese rice samples**

(玄米中ジノテフランの調理過程における減少)

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主論文の要旨

Abstract

Dinotefuran is an insecticide belonging to the neonicotinoid class, which is frequently used to control pests in paddy rice owing to its permeability and effectiveness against sucking insects. Since 2002, this insecticide has been commercially available in Japan, and has become controversial due to its high detection frequency in brown rice for primary consumption. In this study, the effects of processing and cooking on the reduction of dinotefuran residues in commercially available brown rice were investigated. Boiled rice is difficult to homogenize and extract with acetonitrile. Using pre-freezing and cryogenic milling with powdered dry ice, dinotefuran in boiled rice was extracted well. A measurement method comprising sample preparation (acetonitrile extraction, gel permeation chromatography (GPC), and solid phase extraction (SPE)) and detection with a liquid chromatography–tandem mass spectrometry (LC–MS/MS) system was used. In 10 out of 25 commercial brown rice samples, dinotefuran was detected at a concentration of 0.04 µg/g (mean), which was more than the limit of quantitation of 0.01 µg/g. The dinotefuran levels were significantly less than the maximum residue limit of 2 µg/g in Japan. Even after polishing, washing, and boiling, dinotefuran was detected in 10 brown rice samples, with mean residue levels of 74.7%, 60.8%, and 39.6%, respectively, of the original concentration in brown rice. Based on these data, the processing factor of dinotefuran in boiled rice has been estimated to be approximately 0.4. Dinotefuran residues were reduced in the boiled rice, but less so than other pesticides. Although the maximum daily intake of dinotefuran in boiled rice was 0.0065 mg/person/day, its percent ratio to the acceptable daily intake of dinotefuran in Japan was less than 0.05%. These results suggest that the daily intake of dinotefuran from rice might not be a critical problem at present, in spite of its relatively high detection frequency in boiled rice.

要旨

ジノテフランは、ネオニコチノイド系殺虫剤の一種であり、浸透性が高く、吸汁昆虫に対して有効性を示すため、水稻、果実及び野菜などに幅広く使用されている。2002年4月に農薬登録されて以来、その出荷量は年々増加傾向を示しており、玄米中から頻繁に検出される農薬の一つである。今回、ジノテフランが検出された市販玄米を試料とし、精米、米とぎ及び炊飯後のジノテフランの消長を検討した。炊飯白米は、予冷式ドライアイス凍結粉碎法により粉碎・均一化し、酢酸、ヘキサンおよびアセトニトリルにより抽出したのち、GPCおよびSPEにより精製後、LC-MS/MSにて測定する方法を検討した。市販されている玄米25検体中から10検体でジノテフランが検出され（0.01 µg/g、定量下限値）、その平均値は0.04 µg/gであり、残留基準値の2 µg/gと比べて十分に低い値であった。各調理過程後のジノテフランの残存率（%）（玄米を100%として換算）は、白米74.7%、洗米60.8%及び炊飯白米39.6%であった。これらの結果から、炊飯白米の加工係数は0.4と算出した。今回得られた結果と過去の調査結果とを比較したところ、ジノテフランなどの浸透移行性を有する農薬は、非浸透移行性農薬よりも加工調理による減少率が低いことが明らかとなった。炊飯白米からのジノテフランの一日最大摂取量は0.0065 mg/人/日と算出され、ADIと比べると0.05%以下であることから、米からのジノテフラン曝露量は十分に低いものと推定された。

主論文

1. Introduction

Insecticides have a broad spectrum of use in agriculture throughout the world. The newly developed neonicotinoid insecticides, which are absorbed by plants and render the plants toxic to insects for a long time (Wakita et al. 2005), have reduced labour-intensive pesticide spraying with its risk of worker exposure, owing to their low volatility, high permeability, and highly selective insecticidal properties (Jeschke et al. 2011; Casida and Durkin 2013). In general, neonicotinoids are considered good candidates for replacing organophosphorus insecticides, especially in developed countries.

Dinotefuran is a relatively new neonicotinoid insecticide, which was registered in Japan in 2002, and has a unique chemical structure without a chlorine atom or an aromatic ring (Wakita et al. 2003) (Figure 1). Dinotefuran activity differs dramatically from that of other neonicotinoids, with higher activity against sucking insects such as Hemiptera in paddy rice and fruit trees (Tomizawa & Casida 2005). As a result, the domestic shipment of dinotefuran in Japan has dramatically increased in the past decade (Nouyaku-Youran 2017). Therefore, there is growing concern over the residue levels of dinotefuran in food and the health effects of exposure on the human body.

According to the assessment of the pesticide residues levels in brown rice reported by the Japanese government, dinotefuran was detected in 14 of 27 brown rice samples, at less than the maximum residue levels of 2 µg/g in Japan (Japanese Ministry of Agriculture Forestry and Fisheries 2014a; The Japan Food Chemical Research Foundation 2017). This suggests that the continuous intake of dinotefuran from daily dietary intake of rice may be increasing. There is an urgent need to clarify the exposure level of individuals to dinotefuran in daily life. In

particular, it is crucial to obtain practical information about the actual levels of dinotefuran intake from the consumption of boiled rice for the further development of risk assessment. Although rice is the primary food in Japan, limited data are available to estimate the intake levels of dinotefuran from boiled rice, because of the poor understanding of the effect of any cooking process on the reduction in the concentration of dinotefuran in rice.

For this reason, the aim of this study was to understand the reducing effect of cooking on dinotefuran residues in rice samples, after the development of a reliable measurement method for dinotefuran concentration in boiled rice. The following steps are used for preparing boiled rice from brown rice: polishing, washing, and boiling. Therefore, we assessed the concentration of dinotefuran in brown rice, polished rice, washed rice, and boiled rice.

2. Materials and methods

2.1. Solvents and chemicals

Acetonitrile, sodium chloride, ethyl acetate, anhydrous sodium sulfate, acetone, cyclohexane, and n-hexane were of pesticide residue analysis grade (Kanto Chemical, Tokyo, Japan). Methanol, acetonitrile, and water for high-performance liquid chromatography (HPLC) only, were of liquid chromatography mass spectrometry (LC-MS) grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and other reagents were of analytical grade (Wako Pure Chemical Industries, Ltd.). Phosphate buffer (1 mol/L) was prepared by dissolving 105 g of dipotassium hydrogen phosphate and 61 g of potassium dihydrogen phosphate in water and diluting to 1 L.

2.2. Analytical standard

Dinotefuran (purity 99%) was purchased from Wako Pure Chemical Industries. A standard stock solution (1,000 mg/L) was prepared in acetonitrile. A working standard solution was freshly prepared for each assay using methanol. Calibration standard solutions were prepared prior to use by diluting the working standard solution with methanol.

2.3. Apparatus

A rice-polishing machine (MR-E520, Twinbird Company, Niigata, Japan) was used for making polished rice samples. A rice cooker (NL-BS05, Zojirushi, Osaka, Japan) was used to make boiled rice.

An automated gel permeation chromatography (GPC) cleanup system (Shimadzu, Kyoto, Japan) equipped with an CLNpak EV-2000 column (300 × 20 mm i.d., Showa Denko, Tokyo, Japan), CLNpak EV-G guard column (100 × 20 mm i.d., Showa Denko), and ultraviolet (UV) detector was used. An acetone–cyclohexane mixture (1:4) was used as a mobile phase at a flow rate of 5 mL/min. The column oven temperature was 45 °C. The injection volume was 4 mL.

A Nexera LC system (Shimadzu Corporation, Kyoto, Japan) coupled with a QTRAP 4500 MS/MS (AB SCIEX, Framingham, MA, USA), and equipped with an electrospray ionization (ESI) interface, was used to determine dinotefuran. The LC column was a Capcell Pak C18 AQ (150 mm × 2.0 mm i.d., 3 μm, Shiseido Co., Ltd, Tokyo, Japan) with a guard column (Capcell Pak C18 AQ, 10 mm × 2.0 mm i.d., 3 μm). Solvents A and B were ultrapure water with 5 mmol/L ammonium acetate and acetonitrile, respectively. The column temperature was maintained at 40 °C. The injection volume was 3 μL. The MS/MS instrument was operated in the ESI

positive mode at a desolvation temperature of 400 °C and an ESI voltage of 5.5 kV. Tables 1 and 2 summarize the parameters for the LC gradient program and the multiple reaction monitoring (MRM) mode, respectively. The LC gradient program was used for the detection of multiple tagged pesticides.

2.4. Rice samples

Figure 2 shows a flow chart of the procedure to prepare rice samples. Twenty-five brown rice samples were commercially purchased from the Aichi prefecture between 2015 and 2016. First, brown rice (460 g) was polished using a rice-polishing machine and separated into polished rice (415 g) and bran. From this, it follows that approximately 10% (w/w) of the brown rice was removed as bran. Second, the polished rice (142 g) was washed five times with tap water. The polished rice weight after the wash increased to 158 g, owing in part to absorption of water. Finally, the washed rice (158 g) was added to water and boiled for 60 min using an electric rice cooker. This process is a traditional method for rice cooking in Japan.

2.5. Outline of analytical procedure of brown rice, polished rice, and washed rice samples

The concentration of dinotefuran was measured using an LC–MS/MS system according to the previously established method (Ueno et al. 2004; Watanabe et al. 2013). Figure 3 shows a flow chart of the procedure to analyze rice samples. The analytical procedures for brown rice, polished rice, and washed rice samples followed the same steps. Briefly, the sample was first added to water and extracted with acetonitrile, and the extract was separated by a salting-out step. Second, the

acetonitrile layer was collected and evaporated to near dryness, followed by redissolution in ethyl acetate with anhydrous sodium sulfate. Third, the ethyl acetate extract was filtered and evaporated to near dryness, followed by redissolution in acetone-cyclohexane (1:4). Fourth, the extract was loaded on the GPC column, followed by cleaning up of the pesticide fraction using a tandem silica-gel/PSA cartridge solid phase extraction (SPE) column. Finally, the eluate was evaporated to near dryness, and the residue was dissolved in methanol for LC-MS/MS analysis.

2.6. Outline of analytical procedure of boiled rice samples

The concentration of dinotefuran was measured using an LC-MS/MS system according to a previously established method (Watanabe et al. 2013) but with some modifications. Homogenization of the boiled rice was performed by pre-freezing and cryogenic milling with powdered dry ice after cooling to room temperature.

A 10-g sample of the powdered-boiled rice was weighed into a 100-mL centrifuge tube and homogenized for 30 s using 10 mL of 2% acetic acid and 10 mL of n-hexane, and extracted with 25 mL of acetonitrile using a mechanical homogenizer. The extract was centrifuged at 3,000 rpm for 5 min, and the acetonitrile layer was transferred into 100 mL of a graduated cylindrical separatory funnel. The residue and n-hexane layer were re-homogenized using 25 mL of acetonitrile and centrifuged for 5 min at 3,000 rpm. The acetonitrile layer was collected into the same funnel. The combined extract was added to 10 g of ammonium sulfate and shaken for 5 min to salt out the water layer. The half acetonitrile layer extract was evaporated to near dryness. Then, 20 mL of ethyl acetate and 10 g of anhydrous sodium sulfate were added to the residue, and the

resultant mixture was filtered through glass wool. The residue in the flask was rinsed three times using 10 mL of ethyl acetate. The combined filtrate was evaporated to near dryness. The residue was re-dissolved in acetone-cyclohexane (1:4), the volume was adjusted to 10 mL, and the mixture was centrifuged for 10 min at 3,000 rpm. The 4 mL aliquot of the extract was loaded on the GPC system. The GPC fractionation system was used to analyze the total pesticide residues, including neonicotinoid. Fractions 1 (57–75 mL) and 2 (75–160 mL; dinotefuran fraction) were collected. Fraction 1 was cleaned by passing through a tandem SAX/PSA cartridge SPE column (Bond Elut PSA Jr/Bond Elut SAX Jr, 500 mg each, Agilent Technologies, Tokyo, Japan), which was preconditioned with 10 mL of acetone before use. The eluate was combined with fraction 2 and then evaporated to near dryness. The residue was adjusted to 2 mL using methanol. A 100 μ L aliquot of the solution was added to 900 μ L of methanol for (0.1 g of sample/mL) LC–MS/MS analysis.

2.7. Assay validation

Validation tests were performed using brown rice and boiled rice fortified at 0.01 and 0.1 μ g/g. Pesticides at each level were analyzed in two samples per day on 5 days. Validity was evaluated according to the guideline of the Ministry of Health, Labour and Welfare of Japan. The limit of quantitation (LOQ) was calculated as a signal-to-noise ratio (S/N) of 10.

3. Results and discussion

3.1. Extraction of dinotefuran from boiled rice sample

A food processor was used to homogenize the boiled rice, resulting in a paste. It

was difficult to extract dinotefuran from the paste using any solvents. Therefore, the method of preparing powdered-boiled rice by pre-freezing and cryogenic milling with powdered dry ice was adopted in this study.

Figure 4 shows typical chromatograms obtained from blank boiled rice with or without dinotefuran. The developed method to determine dinotefuran in boiled rice afforded high selectivity for determination without any interference peaks, suggesting that cryogenic milling is a robust method to detect dinotefuran in boiled rice without any interference.

3.2. Method validation

The developed method for analyzing boiled rice was validated in terms of selectivity, recovery, and precision according to the guidelines. Table 3 shows the results of the method validation. Mean recoveries were in the range of 95.5–96.8%, and the repeatability (within run) and intermediate reproducibility (between days) in terms of relative standard deviations (RSD%) were within 7.5%. To evaluate the matrix effect in the developed method, the peak areas of the matrix-matched standard solution were compared with appropriate volumes of the standard solution. As the peak area corresponding to the matrix-match standard was the same as that of the standard in a pure solvent, the matrix effect was not observed. The LOQ was estimated as 0.001 µg/g of boiled rice. No interference peaks were observed for the blank sample (Figure 4a). The measurement method of dinotefuran was improved by optimizing pretreatment and LC–MS/MS conditions, resulting in a reliable measurement method for low concentrations of dinotefuran in boiled rice, which has been used in this study (Figures 4(b, c)). High recovery and reproducibility were achieved for the dinotefuran measurement from all rice samples with absolute

calibration curves method, without using an isotope-labeled internal standard.

3.3. Dinotefuran residual levels and the effects of processing and cooking on the concentrations

The dinotefuran residues in commercially available brown rice were surveyed. Table 4 summarizes the concentrations of dinotefuran in 25 brown rice samples. Of these, dinotefuran was detected in 10 brown rice samples at a concentration greater than the LOQ (0.01–0.15 µg/g, mean 0.043 µg/g), which is much lower than the maximum residue limit of 2 µg/g in Japan. This rate of detecting dinotefuran in brown rice is similar to the result reported in a Japanese administrative survey (Japanese Ministry of Agriculture, Forestry and Fisheries, 2014b), indicating that representative sample collection was performed in this study.

Figure 5 shows the effects of polishing, washing, and boiling on the average residual levels of dinotefuran, represented as 100% of dinotefuran in each of the 10 brown rice samples. The concentration of dinotefuran residue after cooking was calculated based on the weight of each sample. The mean residual levels of dinotefuran after polishing, washing, and boiling were 74.7%, 60.8%, and 39.6%, respectively. Based on these data, the processing factor of dinotefuran in boiled rice was estimated to be approximately 0.4.

Table 5 summarizes the Japanese reports, which reported the residual levels of pesticide in polished rice or boiled rice. Saka et al. (2008) comprehensively reported residue levels of several pesticides, and revealed that the residue levels of nine pesticides, including fenitrothion, malathion, and carbaryl, were less than 6% in boiled rice. These results indicated that typical cooking processes for rice are beneficial in decreasing pesticide residues, with different reduction rates. On the

other hand, the dinotefuran residue level in boiled rice was apparently greater than those of other pesticides. We propose the following two reasons. First, dinotefuran can remain inside rice because of its high permeability (Usepa 2004). Second, it is hard to volatilize and is highly stable against high temperatures during boiling. As shown in Figure 6, a negative correlation was observed between the pesticide residue levels in polished rice and the logPow of pesticides, a parameter of water solubility. Although this relationship was not verified in boiled rice, owing to the extremely low residual levels of pesticides other than dinotefuran, the logPow of pesticide is a possible useful parameter for predicting the residue level in boiled rice.

The concentrations of dinotefuran in the 10 brown rice samples ranged from 0.01 to 0.15 $\mu\text{g/g}$. Further work needs to be conducted to obtain practical data to evaluate the actual intake of dinotefuran using a total diet study. The maximum daily intake of dinotefuran in boiled rice was estimated to be 0.0065 mg/person (mean body weight 55.1 kg, daily intake of boiled rice 311.6 g, maximum residue concentration of dinotefuran in boiled rice in this study 0.021 $\mu\text{g/g}$), suggesting that the exposure level of dinotefuran from boiled rice in Japan is considerably less than the acceptable daily intake (ADI) level (0.22 mg/kg body weight/day). According to the ADI, exposure to dinotefuran via boiled rice cannot possibly affect human health at this time. Although the measurement samples were only 25 brands obtained from various Japanese regions, this study provided scientific information for understanding the possible risks to human health of exposure to dinotefuran. Further studies are needed to understand the acceptable exposure level to dinotefuran for children and pregnant women, who are vulnerable to chemical exposure and for whom rice is an important food.

4. Conclusions

This study is the first report on the characteristics of dinotefuran residue levels during the cooking process of rice, which is the staple food of Japan. Even after rice samples were cooked, residual levels of dinotefuran of approximately 40% were observed, because of high permeability. Among 10 commercially purchased samples, the maximum residue concentration of dinotefuran in boiled rice was 0.021 µg/g. This basic information related to the residual level of dinotefuran in rice samples will contribute to the development of a more reliable evaluation of dinotefuran exposure levels with respect to human health.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Figure legends

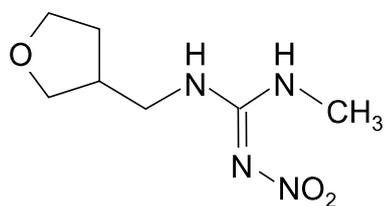


Figure 1. Structure of dinotefuran.

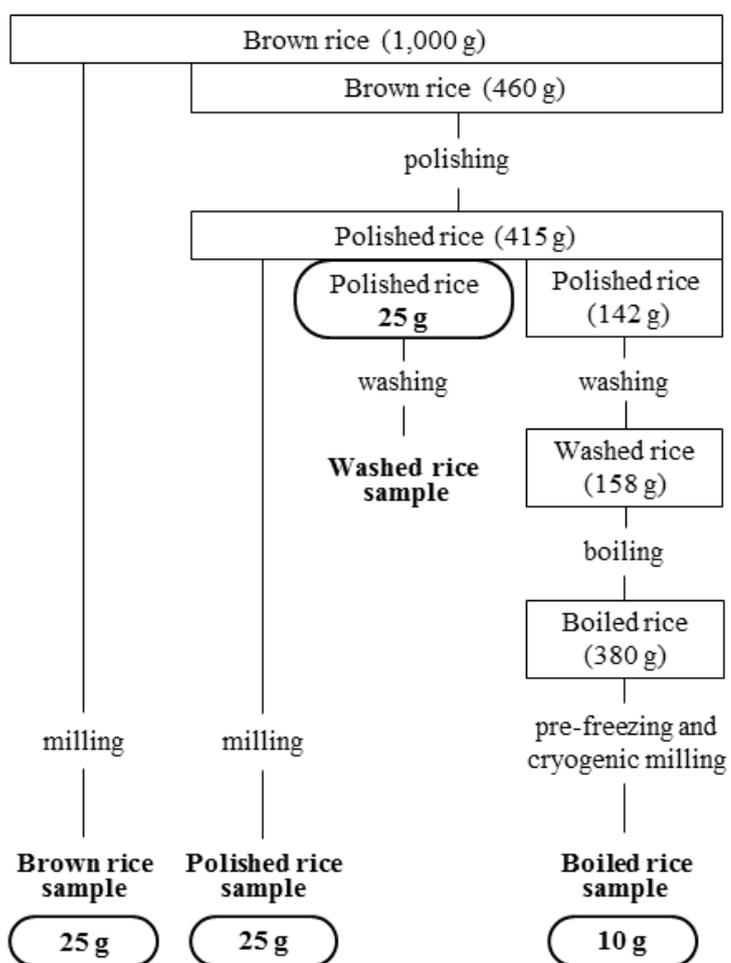


Figure 2. Outline of preparation procedure of rice samples.

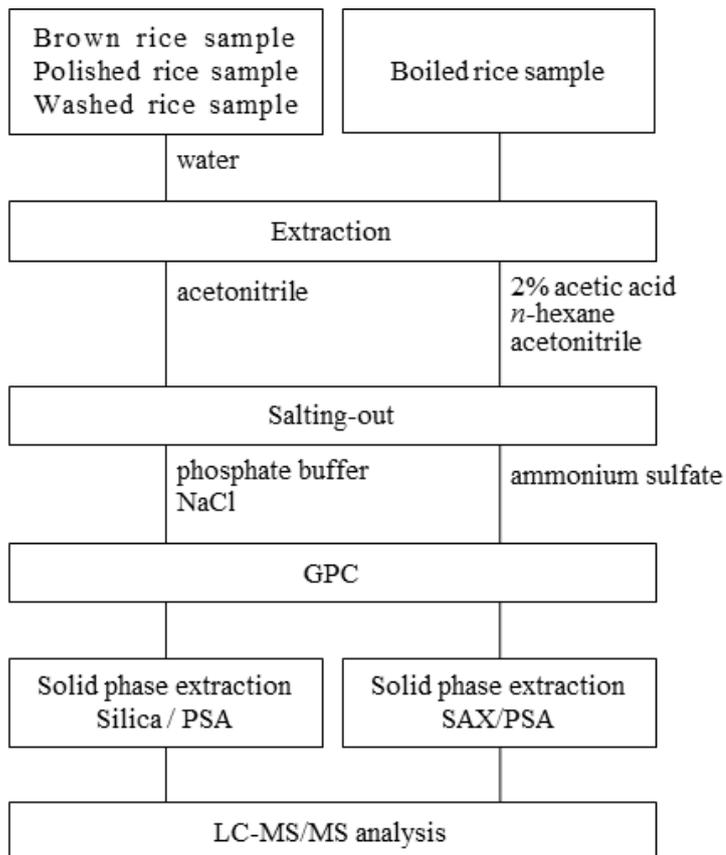


Figure 3. Outline of analysis procedure of rice samples.

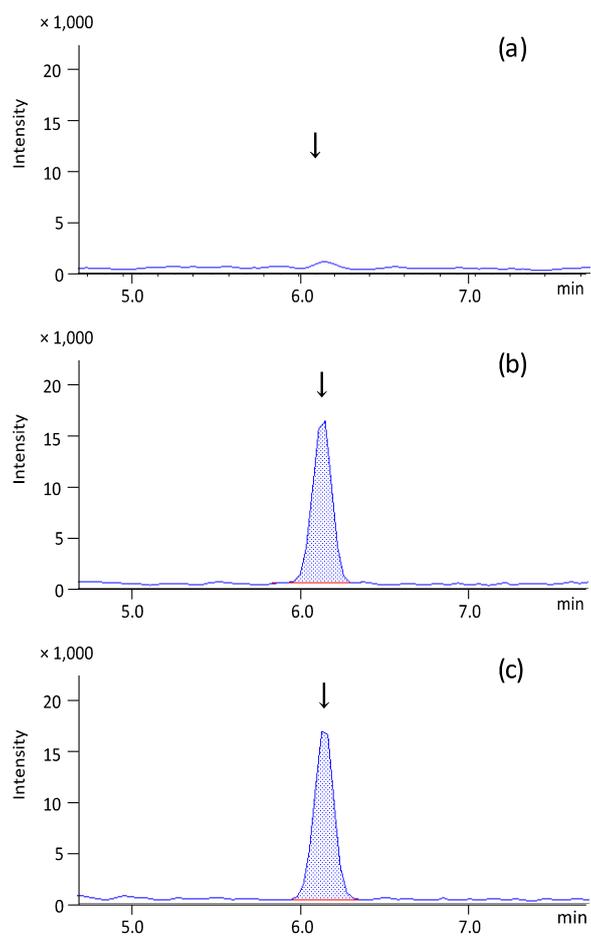


Figure 4. LC-MS/MS chromatograms of (a) blank boiled rice, (b) boiled rice fortified with dinotefuran at $0.01 \mu\text{g/g}$, (c) standard solution of dinotefuran ($0.001 \mu\text{g/mL}$)

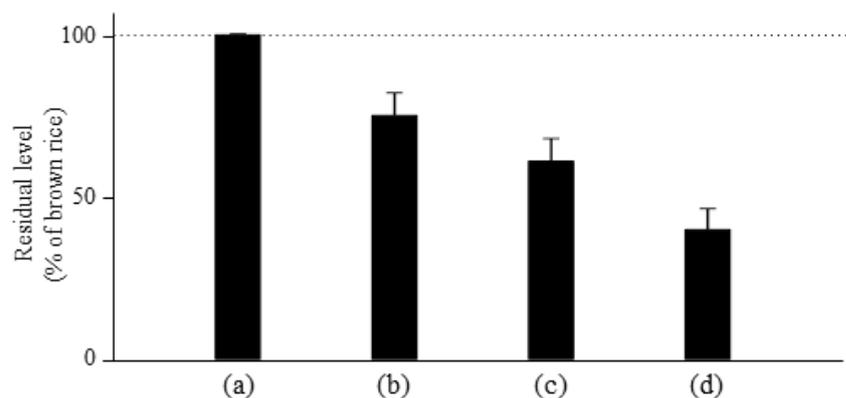


Figure 5. Residual levels of dinotefuran in rice products.
 (a) brown rice, (b) polished rice, (c) washed rice, (d) boiled rice.

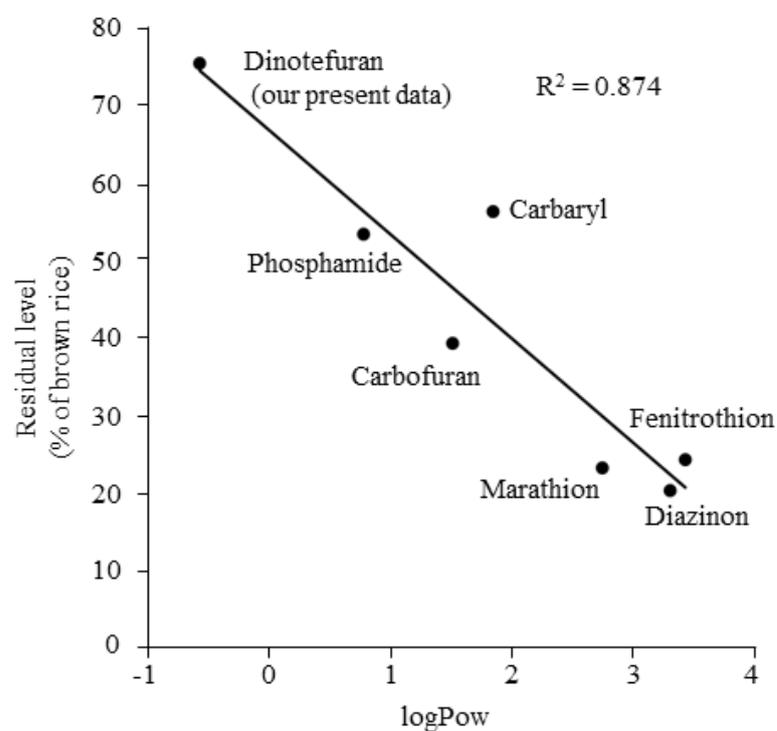


Figure 6. Relationship between logPow and residual level of pesticides in polished rice. This figure was composed of previous reported data Saka et al (2008) and our data.

Table legends

Table 1. LC gradient parameters for the elution of the dinotefuran

Mobile phase			Flow rate	
Time (min)	A (%)	B (%)	Time (min)	Flow rate (mL/min)
0	99	1	0	0.2
1	99	1	20	0.2
1.01	90	10	40	0.35
25	5	95		
26	1	99		
40	1	99		

A; ultrapure water with 5 mmol/L ammonium acetate. B; acetonitrile

Table 2. MRM parameters

DP* ¹ (V)	Target ion			Qualifier ion		
	MRM trace (<i>m/z</i>)	CE* ² (V)	CXP* ³ (V)	MRM trace (<i>m/z</i>)	CE* ² (V)	CXP* ³ (V)
49	203 > 113	15	7	203 > 129	15	7

*¹ Declustering potential

*² Collision energy

*³ Collision cell exit potential

Table 3. Recovery rates, repeatability, and reproducibility of determination method of dinotefuran in boiled rice

Sample	Spiked level ($\mu\text{g/g}$)	Recovery (%)	Repeatability (RSD%)	Reproducibility (RSD%)
Boiled rice	0.1	95.5	2.3	7.5
	0.01	96.8	3.2	4.8

Table 4. Dinotefuran concentration in brown rice

Brown rice sample ID	Concentration ($\mu\text{g/g}$)	Brown rice sample ID	Concentration ($\mu\text{g/g}$)
1	0.010	13	n.d.
2	0.012	14	0.034
3	Tr* ¹	15	Tr
4	0.147	16	Tr
5	Tr	17	Tr
6	Tr	18	Tr
7	n.d.* ²	19	0.013
8	0.036	20	0.046
9	0.014	21	n.d.
10	0.013	22	Tr
11	Tr	23	n.d.
12	Tr	24	0.103
		25	n.d.

*¹ Trace level (Tr) of between 0.005 and less than 0.009 $\mu\text{g/g}$ could be confirmed.

*² Not detected

Table 5. Summary of previously reported residual levels of pesticides in polished rice and boiled rice

Reports	Sample	Pesticide	Residual level (% of brown rice)	
			Polished rice	Boiled rice
Satoh et al. 2003	Pesticides were sprayed in a paddy field	Fenobucarb	26	9
		Flutoranil	25	11
Ueno et al. 2006	Brown rice was bought in the retail store	37 kinds of pesticides	19 (mean)	8 (mean)
Saka et al. 2008	Pesticides were sprayed in a paddy field	Fenitrothion	24	3.1
		Daiazinon	20	6.4
		Carbaryl	56	1.9
Our present data	Brown rice was bought in the retail store	Dinotefuran	74.7	39.6