A Single Residue Method for the Determination of Chlorpropham in Representative Crops Using High Performance Liquid Chromatography

Hyeri Lee · Myoungjoo Riu · Eunhye Kim · Joon-Kwan Moon · Hoon Choi · Jung-A Do · Jae-Ho Oh · Ki-Sung Kwon · Young Deuk Lee · Jeong-Han Kim

Abstract A single residue analytical method was developed for herbicide chlorpropham in various crops. Brown rice, apple, mandarin, Kimchi cabbage, green pepper, potato, and soybean were selected as representative crops, and clean-up system, partition solvent, and extraction solvent were optimized. For high performance liquid chromatography (HPLC), C_{18} column was used with elution solvents of water and acetonitrile. Limit of quantitation (LOQ) of chlorpropham was 2 ng (S/N >20), and excellent linearity ($R^2 = 1.000$) was achieved. Method limit of quantitation (MLOQ) was 0.02 mg/kg. For recovery tests, crop samples were macerated and fortified with chlorpropham standard solution at three fortification levels (MLOQ, 10 MLOQ, and 100 MLOQ). And then those were extracted with acetonitrile, concentrated and partitioned with n-hexane. The n-hexane layer was then concentrated, cleaned-up through Florisil® column with ethyl acetate:n-hexane (5:95, v/v) prior to concentration and analysis with HPLC. Good recoveries from 76.8 to 107.9% with coefficients of variation of less than 10% were obtained, regardless of sample type, which satisfies the criteria of Korea Food and Drug Administration. Those results were confirmed with liquid chromatography-mass spectrometry (LC-MS). The method established in this study could be applied to most of crops as an official and general method for the analysis of chlorpropham residue.

Introduction

Although crops are main food sources, they are susceptible to various diseases, weeds, insects, and nematodes. Therefore, pesticides have been used to protect food crops from these pests (Ware and Whitacre, 2004). However, pesticide residue on the crops/food is the representative issue in food safety aspects, because pesticides have intrinsic toxicity to a certain level. To guarantee consumer safety and international trade, acts and regulations were established in many countries to control pesticide residues in food products (Ahmed, 2001), and maximum residue limits (MRLs) in crops/food have been set by government agencies. As a typical example, Korea Food and Drug Administration (KFDA, 2012) established 11,696 MRLs of 427 pesticides and 367 crops/food for monitoring pesticide residues in food, many analytical methods have been employed. Analysis of pesticide residues is very difficult due to complex sample matrices, many types of pesticides, and low levels of contamination. Therefore, analytical methods should be able to measure at very low levels of residue with the highest reliability for identity and concentration in crops/food (Taylor et al., 2002).

Chlorpropham (isopropyl 3-chlorocarbanilate; Fig. 1A) is a carbamate herbicide with low mammalian toxicity (LD_{50} for rats:

H. Lee · M. Riu · E. Kim · J.-H. Kim (✉)
Department of Agricultural Biotechnology, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151-742, Republic of Korea
E-mail: kjh2404@snu.ac.kr

J.-K. Moon
School of Plant Life and Environmental Sciences, Hankyong National University, Ansung 456-749, Republic of Korea

H.Choi · J.-A. Do · J.-H. Oh · K.-S. Kwon
National Institute of Food and Drug Evaluation, Osong 363-951, Republic of Korea

Y. D. Lee
Division of Life and Environmental Science, Daegu University, Gyeongsan 712-714, Republic of Korea

4200 mg/kg) and ecological effect (Log P_{ow}; 3.79 at 20°C, pH 4). It is degraded in soil [50% dissipation time (DT_{50}) in soil: 30–65 days] by microbial degradation and then converted into 3-chloroaniline by an enzymic hydrolysis reaction (Tomlin, 2009). Chlorpropham was registered in Korea in 1995 (Korea Crop Protection Association, 2010), and its MRLs were established for more than 70 crops at the level of 0.05–50 mg/kg (Korea Food and Drug Administration, 2012).

Many analytical studies for chlorpropham residue determination were reported from some plant matrices; however, such methods are considered not to be proper for standard analytical method, because the subject crops are limited, not validated in full manner, and need too much time and labor due to many steps required for sample preparation (Lentza-Rizos and Balokas, 2001; Orejuela and Silva, 2004; Sakaliene et al., 2009). In Korea, the analytical method of chlorpropham residues in crops/food is listed in Food Code (Korea Food and Drug Administration, 2012) as an individual residue method using gas chromatography-nitrogen phosphorous detector/flame photometric detector (GC-NPD/FPD). However, in the separation of chlorpropham, old type of packed column was used, which is no longer used in residue analysis, and method validation data were not available; thus, such method is not suitable for use as a precise and reliable standard analytical method.

The purpose of this study is the establishment of the standard analytical method for detection of chlorpropham residues in crops/food, which can be applied generally and officially to many different samples. Method validation was performed, and improvement for more efficient and simpler clean-up procedures than the other existing methods was achieved. Seven representative crops were selected from five crop groups.

Materials and Methods

The subject pesticides and crops. Standard material of chlorpropham (98.4%) (Fig. 1A) was purchased from Fluka™ (Switzerland). Brown rice, apple, mandarin, kimchi cabbage, green pepper, potato, and soybean of "residue-free grade" were purchased from a local market. They were chopped, macerated, and kept in a freezer at a temperature below –20°C in polyethylene bags.

Chemicals, reagents and standard solutions. Acetonitile, acetone, *n*-hexane, and ethyl acetate were HPLC grade (Burdick and Jackson®, Korea). Sodium sulfate (GR grade) and sodium chloride (GR grade) were from Junsei Chemical Co. Ltd. (Japan). Florisil® (60-100 mesh) was purchased from Fluka™ and activated by drying at 130°C for over 5 h. Filter papers (GF/A) were from Whatman International Ltd. (England). Diethylene glycol was purchased from Sigma-aldrich (USA). A stock solution of chlorpropham was prepared in acetonitrile at a concentration of 1000 mg/L, and the working solutions were prepared by appropriate dilutions of the stock solutions with acetonitrile.
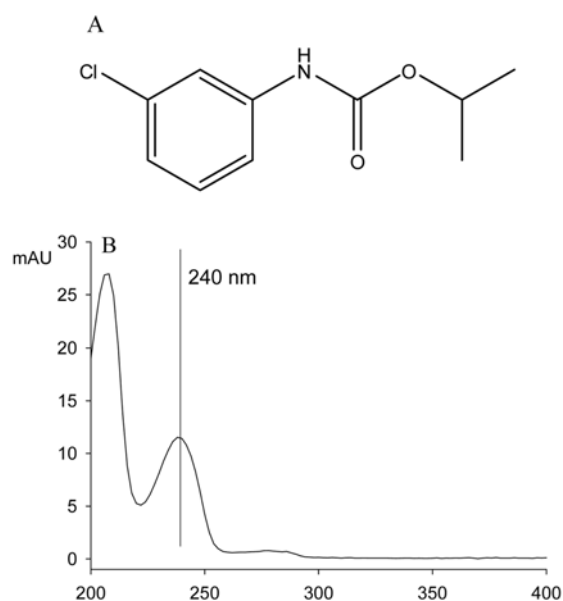

Fig. 1 Structure (A) and UV spectrum (B) of chlorpropham.

Measurement of instrumental sensitivity and calibration curve linearity. Chlorpropham standard solutions (1, 0.1, and 0.05 mg/L) were analyzed with HPLC, and the Signal to Noise ratio (S/N) of chlorpropham peak on chromatograms was calculated for LOD (S/N >3) and LOQ (S/N >10). The standard solutions at concentrations of 0.05, 0.1, 0.5, 1, and 5 mg/L were analyzed with HPLC, and calibration curve linearity (R^2) was measured.

Establishment of the HPLC condition for the separation of chlorpropham in crop samples. HPLC analysis was performed using an Agilent HPLC 1100 series system with YMC-Pack pro C18 column (250 mm × 4.6 mm i.d., 5 μm particles, Japan), and column temperature was maintained at 35°C. For the analysis of brown rice, apple, mandarin, kimchi cabbage, and potato samples, the mixture of acetonitrile and water (55:45, v/v) was used as mobile phase (flow rate; 1.0 mL/min), and gradient elution was used for green pepper and soybean samples (flow rate; 1.5 mL/min), which gave more complex matrix peaks. The initial composition (45% acetonitrile) was kept for 13 min, and then a linear gradient to 80% acetonitrile was programmed in 20 min, with a final hold of 3 min. The initial condition was recovered in 3 min with a hold for 1 min before next run. The injection volume was 20 μL, and the detection wavelength of chlorpropham in crop samples was at 240 nm. For the selection of optimum detection wavelength of chlorpropham, a full UV spectrum with diode-array detector (DAD; 200-400 nm) was obtained by analyzing an aliquot (20 μL) of a standard solution (1 mg/kg) by HPLC under isocratic elution (acetonitrile:water = 70:30).

Establishment of sample preparation procedure for chlorpropham. In establishment of the optimum clean-up system, a glass column (35 cm × 1.5 cm i.d.) was filled with activated

Florisil® (10 g), and anhydrous sodium sulfate (3 g) was added on top. The column was then conditioned with *n*-hexane (100 mL) before loading the chlorpropham standard solution (5 mL, 1 mg/L). The column was eluted with 50 mL of 5, 10, 15, 20, 25% ethyl acetate/*n*-hexane mixture in sequence. Each eluate was evaporated at 35ºC with 200 μL of 2% ethylene glycol to dryness, and the residue was dissolved with acetonitrile (5 mL) before analysis with HPLC.

For the optimization of the liquid-liquid partitioning system, an aliquot of chlorpropham solution (1 mL, 5 mg/L) was added to water (25 mL) and left standing for about 30 min in a separatory funnel before water (50 mL) and saturated sodium chloride solution (50 mL) were added. The mixture was extracted two times with each portion of three different solvents (dichloromethane, *n*-hexane and ethyl acetate; 100 and 50 mL for each solvent). Each organic phase was dried over anhydrous sodium sulfate and evaporated under 35ºC with 200 μL of 2% ethylene glycol for concentration. The residue was dissolved with acetonitrile (5 mL) and analyzed with HPLC.

Recovery test of chlorpropham in crop samples. Samples (25 g) of brown rice, apple, mandarin, kimchi cabbage, green pepper, soybean, and potato were macerated and fortified with chlorpropham standard solution at 0.02 mg/kg (MLOQ), 0.2 mg/kg (10MLOQ) and 2 mg/kg (100MLOQ) levels before they were extracted by shaking at 180 rpm for 1 h with acetonitrile (100 mL). The mixture was filtered through a Whatman™ GF/A filter paper, and the filter cake was rinsed with acetonitrile (30 mL). The filtrate was concentrated under reduced pressure at 35ºC with 200 μL of 2% ethylene glycol. The concentrate was dissolved in *n*-hexane (100 mL), water (50 mL) and saturated sodium chloride solution (50 mL) for partitioning by shaking. Partitioning was repeated once more with 50 mL of *n*-hexane. The combined *n*-hexane layer was dried over anhydrous sodium sulfate and concentrated, and the residue was dissolved in *n*-hexane (5 mL). After loading the extract on the Florisil® column, which was conditioned with *n*-hexane (100 mL), the column was washed with 100 mL of *n*-hexane and eluted with 100 mL of ethyl acetate/*n*-hexane (5:95, v/v). The eluate was concentrated, dissolved with acetonitrile (5 mL), and analyzed with HPLC.

Retention factor of chlorpropham on chromatogram. Retention factor (capacity factor, k) was calculated using retention time (t_r) and adjusted retention time (t_r') (Rood, 2007).

$k = t_r'/t_m$
t_r = retention time (min)
t_m = retention time of a non-retained compound (min)
$t_r' = t_r-t_m$ = adjusted retention time (min)

Number of theoretical plate (N) and height equivalent to a theoretical plate (H). N was calculated using t_r and peak width. N and column length was used for calculation of H (Rood, 2007).

$N = 5.545(t_r/W_h)^2$
W_h = peak width at half height
H (mm) = column length (mm)/N

Analysis of crop samples by LC-MS. LC-MS (Agilent 1100

Table 1 MRLs of chlorpropham in various crops (Korea Food and Drug Administration, 2012)

Group	Number of crops	MRLs
Grains	9	0.05–1
Fruits	23	0.05
Vegetables	21	0.05–0.2
Potatoes	3	0.05–50.0
Beans and oily crops	15	0.05–0.2
Others	1	0.05

series) connected with Agilent 1100 HPLC was used for confirmation of residue analysis. For HPLC, Imtakt Unison US-C18 column (2.0 mm × 150 mm i.d., 5 μm particles) was used with elution solvent of acetonitrile and 0.1 mM ammonium acetate solution (50:50; v/v) (flow rate: 0.2 mL/min).

For the best formation of its protonated molecular ion ([M+H]⁺; *m/z* 214), MS fragmentor voltage was optimized by analyzing a standard solution (1 mg/kg) of chlorpropham (2 μL) by LC-MS with electrospray ionization (ESI) in positive mode (mass range: *m/z* 100-240). Subsequently, *m/z* 214 ion was used as a selective ion monitoring (SIM) ion in analysis of crop samples (0.2 mg/kg level). Drying gas temperature and fragmentor voltage were 350ºC and 50 volts, respectively.

Results and Discussion

The representative crops and MRLs. Since MRLs of chlorpropham were established for a variety of crops (Table 1), brown rice (grains), apple, and mandarin (fruits), kimchi cabbage and green pepper (vegetables), potato (potatoes), and soybean (beans and oily crops) were selected as the representative crops considering their popularity and matrix characteristics in analytical aspects for application of developed method to most of crops.

HPLC detection. Optimum detection wavelength of chlorpropham was investigated for sensitive determination. When a full UV spectrum of chlorpropham was recorded using DAD, λ_{max} was observed at 205 and 240 nm (Fig. 1B); however, 240 nm was used as a detection wavelength due to its similarity to those of other reports (Camire et al., 1995; Basheer et al., 2009; Paiga et al., 2009; Sakaliene et al., 2009).

Clean-up method with Florisil® column chromatography. In pesticide residue analysis, adsorption chromatography is generally used for clean-up of the interfering coextractives (e.g. lipids and pigments), which were not removed by liquid-liquid partitioning (Fong et al., 1999). In the present study, Florisil®, the most popular sorbents material for clean-up in general pesticide analysis, was chosen for absorption column chromatography, whereas some reports used several materials, such as, alumina N (Lentza-Rizos and Balokas, 2001), active carbon (Orejuela and Silva, 2004), Oasis-HLB SPE (Camire et al., 1995), and C18 (de Carvalho et al., 2009). Various combinations of ethyl acetate/*n*-hexane were

Table 2 Recovery rate by sequential elution with ethyl acetate/*n*-hexane

Ethyl acetate/*n*-hexane		Recovery (%)
v/v	Volume	
5 : 95	50 mL	47.3
10 : 90	50 mL	53.3
15 : 85	50 mL	-
20 : 80	50 mL	-
25 : 75	50 mL	-
Total		100.6

eluted in sequence with 50 mL of 5, 10, 15, 20, and 25% ethyl acetate/*n*-hexane mixture after loading of chlorpropham extract on Florisil® column. As a result, mixtures of 5:95 and 10:90 (ethyl acetate/*n*-hexane, v/v) gave good recoveries. Therefore, the mixture of 100 mL of 5:95 combination was chosen as the elution solvent, whereas 100 mL of *n*-hexane was used for washing the column to remove early eluting impurities without losing chlorpropham (Table 2).

Liquid-liquid partitioning of chlorpropham. After establishment of clean-up procedure successfully as mentioned above, liquid-liquid partitioning system was examined, because immiscible solvents, such as water versus organic solvents removes the polar interfering coextactives (e.g. carbohydrates) (Fong et al., 1999). Sodium chloride was added in partitioning system, because more 'salt' dissolves in the aqueous phase, more of the pesticide is partitioned into the organic phase (Fong et al., 1999). In the present study, three organic solvents such as dichloromethane, ethyl acetate, and *n*-hexane were used with water. Although chlorpropham was well partitioned with three solvents (97.6% at *n*-hexane; 96.7% at ethyl acetate; 102.6% at dichloromethane), *n*-hexane (100 and 50 mL) was selected as the partitioning solvent, because it does not extract polar impurities.

Method validation. Method validation is a set of procedures to evaluate the performance characteristics such as recovery, linearity and range of calibration, limits of detection (LOD), and quantitation (LOQ) of a method for specific analyte and sample types (Fong et al., 1999; Codex Alimentarius Commission, 2003; Miller, 2005). From the analysis of several concentrations, 0.4 and 2 ng was determined as LOD and LOQ (S/N ratio >20), respectively, which are satisfactory for sensitive analysis of chlorpropham residue. The calibration curve was constructed with the mean value of triplicate analysis of chlorpropham standard solutions at the

concentration between 0.05 and 5 mg/kg. The regression equation was y=101.4433x–0.5552 with excellent coefficients of determination (R^2=1.000).

MLOQ (Method Limit of Quantitation; mg/kg) is a practical LOQ of the total analytical method and is usually calculated using LOQ, injection volume, final extract volume and sample weight in analytical method (Lee et al., 2008, Lee et al., 2012).

MLOQ=(LOQ × final extract volume)/(injection volume × sample weight)   (1)

MLOQ value for chlorpropham as calculated using the equation 1 was 0.02 mg/kg. This value satisfied the criteria of KFDA, which are below 0.05 mg/kg or half of MRL (Lee, 2011). Compared to other works with crisps (MLOQ=0.035 mg/kg) (Ritchie et al., 1983), this result is far more sensitive.

Recovery test can provide efficiency of sample preparation method by recovered rate (accuracy, %) and coefficient of variation (precision, %) (Fong et al., 1999). Untreated samples were spiked with chlorpropham standard solutions at the concentration of MLOQ, 10MLOQ, and 100MLOQ levels. The sample preparation and analysis were performed using the established method in the present study to give reasonable recoveries (76.8–107.9%) and low coefficient of variation (0.3–7.3%) (Table 3 and Fig. 2). Orejuela and Silva (2004) reported 97.6–102.2% of recoveries with potato, whereas de Carvalho et al. (2009) reported that recovery was 113.3–129.9% with medicinal plants.

Retention factor (*k*) and chromatographic efficiency in resolution of chlorpropham on HPLC. Retention factor (partition ratio or capacity factor; *k*) measures the extent to which a solute (chlorpropham) is retained (Miller, 2005; Rood, 2007; Lee, 2011; Lee et al., 2012) and was found to be 5.29 and 3.37 for chlorpropham (Table 4), indicating enough retention for good separation.

Plate number (*N*) and plate height (*H*) express the efficiency of a column (Miller, 2005; Rood, 2007; Lee, 2011), indicating high efficiency columns have large *N* and small values of *H*. For chlorpropham *N* was 12877 and 8545; thus, *H* was 0.019 and 0.029 mm in each crop sample (Table 4), suggesting high column efficiency.

Confirmation of chlorpropham in crop matrices by LC-MS. LC-MS was used to confirm the chlorpropham residue in the fortified recovery crop samples, because when a certain pesticide

Table 3 Recovery and MLOQ for chlorpropham in crops

Fortified level (mg/kg)	Recovery (accuracy, %)[a] / coefficient of variation (precision, %)[b]							MLOQ (mg/kg)
	Brown rice	Apple	Mandarin	Kimchi cabbage	Green pepper	Potato	Soybean	
0.02	96.2/2.6	99.7/0.8	93.8/1.6	107.9/0.3	83.0/2.3	99.0/3.2	88.9/5.1	
0.2	93.0/1.0	91.2/0.6	88.8/2.2	88.4/1.6	84.7/1.4	88.9/2.0	77.3/0.8	0.02
2	90.4/1.4	88.4/5.1	87.4/0.9	83.1/2.8	89.0/4.8	81.0/0.2	76.8/7.3	

[a]Average of triplicate
[b]Coefficient of variation, standard deviation/mean×100
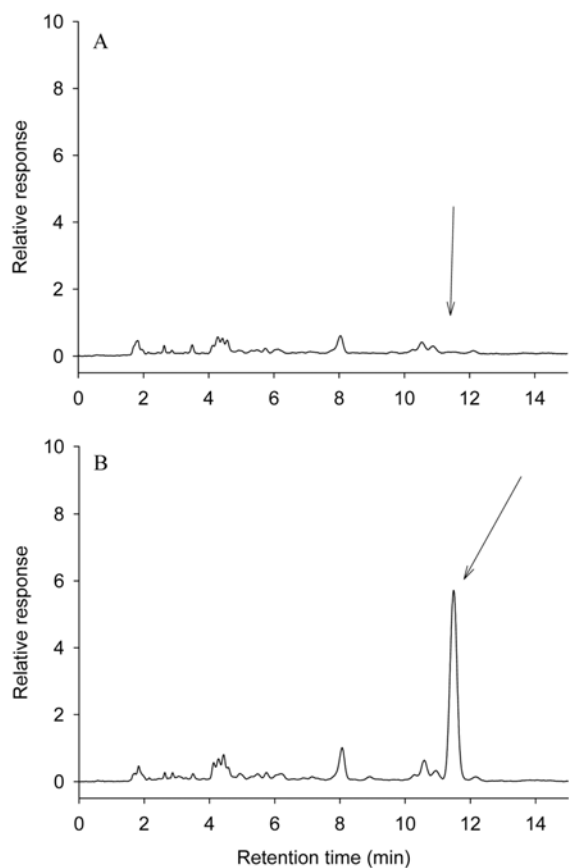
Fig. 2 Representative chromatograms of control (A) and recovery (B) of chlorpropham of mandarin extracts (0.2 mg/kg level).

Fig. 3 LC-MS total ion chromatogram (A) and full scan mass spectrum (B) of chlorpropham.

residue is found in survey/monitoring samples, its identity must be confirmed unambiguous by MS using an official procedure (Lee, 2011). Full scan spectrum of chlorpropham gave m/z 214 ion, the protonated molecular ion $[M+H]^+$, as the most intensive peak (Fig. 3) after fragmentor voltage was tuned to optimum value. Ion m/z 216 was also observed due to one chlorine atom in a molecule with 1/3 intensity of m/z 214 ion. Analysis by LC-MS with SIM mode using m/z 214 ion confirmed that the corresponding peak in recovery samples is a true chlorpropham residue.

In conclusion, new or improved analytical methods were established for chlorpropham residue in brown rice, apple, mandarin, kimchi cabbage, green pepper, soybean, and potato using HPLC. By full method validation, the method achieved good accuracy and precision, and the results were confirmed with LC-MS(SIM), indicating that the established method can be used as the reliable official method for most of the crops.
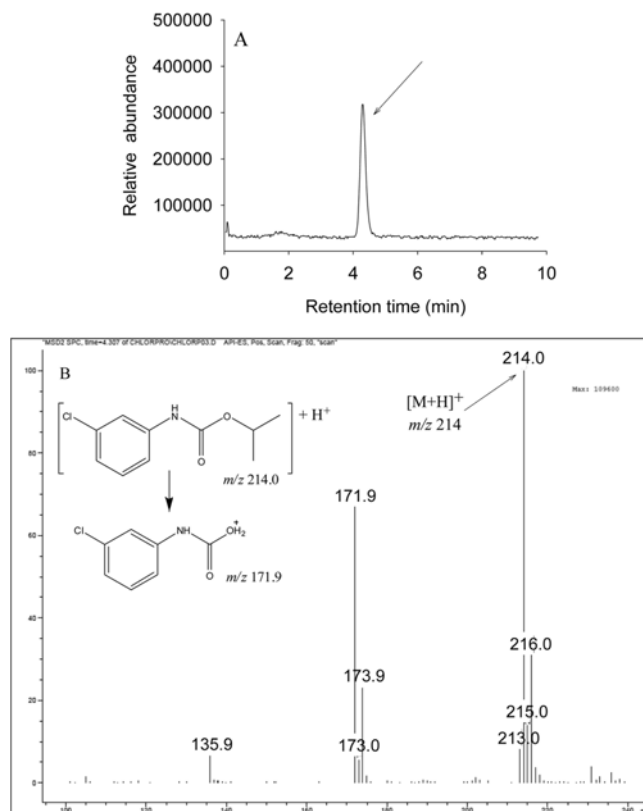
References

Ahmed FE (2001) Analyses of pesticides and their metabolites in foods and drinks. *Trac-Trends in Analytical Chemistry* **20**, 649–61.

Basheer C, Alnedhary AA, Rao BSM, and Lee HK (2009) Determination of carbamate pesticides using micro-solid-phase extraction combined with high-performance liquid chromatography. *Journal of Chromatography A* **1216**, 211–6.

Camire ME, Bushway RJ, Zhao JX, Perkins B, and Paradis LR (1995) Fate of Thiabendazole and Chlorpropham Residues in Extruded Potato Peels. *Journal of Agricultural and Food Chemistry* **43**, 495–7.

Codex Alimentarius Commission (2003) *Guidelines on good laboratory practice in residue analysis*. p. 5-7, 13–29, Nation.

de Carvalho PHV, Prata VD, Alves PB, and Navickiene S (2009) Determination of Six Pesticides in the Medicinal Herb Cordia salicifolia by Matrix Solid-Phase Dispersion and Gas Chromatography/Mass Spectrometry. *Journal of Aoac International* **92**, 1184–9.

Fong WG, Moye HA, Seiber JN, and Toth JP (1999) In *Pesticide residues in*

Table 4 Retention times (t_r), retention factor (k), number of plates (N) and height of theoretical plate (H) of chlorpropham (each analytical condition)

Crops	t_r (min)	t_m (min)	t_r'	K	N	H (mm)
Brown rice, apple, mandarin, Kimchi cabbage, and potato	11.416	1.815	9.60	5.29	12877	0.02
Green pepper and soybean	8.397	1.922	6.48	3.37	8545	0.03

foods: methods, techniques, and regulations. pp. 3-22, 40–2, Wiley, New York.

Korea Crop Protection Association (2010) *Pesticide handbook.* p. 54, Korean Crop Protection Association, Nation.

Korea Food and Drug Administration (2012) *Food Code.* p. Attached table 4–37, KFDA, Nation.

Lee H, Kim E, Moon JK, Zhu YZ, Do JA, Oh JH et al. (2012) Establishment of Analytical Method for Cyazofamid Residue in Apple, Mandarin, Korean Cabbage, Green Pepper, Potato and Soybean. *Journal of the Korean Society for Applied Biological Chemistry* **55**, 241–7.

Lee JH, Park HW, Keum YS, Kwon CH, Lee YD, and Kim JH (2008) Dissipation pattern of boscalid in cucumber under greenhouse condition. *The Korean Journal of Pesticide Science* **12**, 67–73.

Lee YD (2011) In *Practical Guide for Food Code Pesticide Residue Analysis.* Revised ed. (ed.), pp. 38-43, 75–7, 79, KFDA, Osong, Korea.

Lentza-Rizos C and Balokas A (2001) Residue levels of chlorpropham in individual tubers and composite samples of postharvest-treated potatoes. *Journal of Agricultural and Food Chemistry* **49**, 710–4.

Miller JM (2005) In *Chromatography: concepts and contrasts.* 2nd (ed.), pp. 24–29, 45–55, Wiley, Hoboken, N.J.

Orejuela E and Silva M (2004) Determination of propham and chlorpropham in postharvest-treated potatoes by liquid chromatography with peroxyoxalate chemiluminescence detection. *Analytical Letters* **37**, 2531–43.

Paiga P, Morais S, Correia M, Delerue-Matos C, and Alves A (2009)

Determination of carbamate and urea pesticide residues in fresh vegetables using microwave-assisted extraction and liquid chromatography. *International Journal of Environmental Analytical Chemistry* **89**, 199–210.

Ritchie W, Boyd IMG, and Duncan HJ (1983) A Method for the Determination of Chlorpropham Residues in Crisps and Crisp Frying Oil. *Potato Research* **26**, 73–7.

Rood D (2007) In *The troubleshooting and maintenance guide for gas chromatographers.* 4th, rev. and updated (ed.), pp. 14–22, Wiley-VCH, Weinheim.

Sakaliene O, Koskinen WC, Blazauskiene G, and Petroviene I (2009) Level and fate of chlorpropham in potatoes during storage and processing. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes* **44**, 1–6.

Taylor MJ, Hunter K, Hunter KB, Lindsay D, and Le Bouhellec S (2002) Multi-residue method for rapid screening and confirmation of pesticides in crude extracts of fruits and vegetables using isocratic liquid chromatography with electrospray tandem mass spectrometry. *Journal of Chromatography A* **982**, 225–36.

Tomlin C (2009) In *The pesticide manual: a world compendium.* 15th (ed.), pp. 184–5, British Crop Protection Council, Alton, Hampshire.

Ware GW and Whitacre DM (2004) In *The pesticide book.* 6th (ed.), pp. 3–5, Meister Media Worldwide, Willoughby, OH.