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Original research article

Chemical Hazard Assessment and Proposed Management Methods for Safe Drone Pupae (*Apis mellifera* L.) Production

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Abstract In this study, 40 samples from different stages of drone pupae production were analyzed to assess the corresponding chemical hazards and to seek chemical hazard management measures for safe drone pupae production. Drone pupa, beehive, and imported pollen were collected from domestic farms. Domestic pollen was purchased online from farms. The samples were evaluated for mycotoxins, veterinary drugs, residual pesticides, and heavy metals. No mycotoxins were detected in all samples. As for veterinary drugs, berberine was detected in domestic pollen while trimethoprim was detected in imported pollen, but both components were not included in the honey residual standards of the Food Code and within acceptable level, even when standards for other livestock species were applied. Residual pesticides were detected in beehives, domestic pollen and imported pollen, which were all within appropriate levels for the residue standards of agricultural and livestock products in the Food Code. However, cyprodinil noted in beehive, was not included as pesticide residue standard in honey and exceeded the standard values for pesticide residue in other livestock products. Heavy metals were observed in all samples, but all appeared to meet criteria limits. Management of pesticides and veterinary medicines from the nearby environment should be strictly done to prevent chemical hazards from getting into drone pupae during production. Additionally, checking the inspection report when receiving raw and subsidiary materials and conducting a residual substance inspection after collection should also be properly implemented for safe drone pupae production.

Keywords Chemical hazard analysis, Drone pupae, Edible insects, HACCP, Mycotoxin

INTRODUCTION

Due to climate change, concerns related to food such as crop cultivation and livestock breeding are increasing, and interest in new food resources is increasing in preparation for future food shortages. Edible insects with environmental benefits such as high feed efficiency, small water requirement for production, as well as low environment impact due to no methane by-product during the production process, are attracting attention (Baiano, 2020). The Ministry of Agriculture, Food and Rural Affairs (2022) announced that the number of farms and corporations reporting insect business in Korea in 2021 was 3,012, a 4.8% increase from 2,873 farms in 2020. In addition, insect sales in 2021 were KRW 44.6 billion, an increase of 7.7% compared to KRW 41.4 billion in 2020. There are a total of 10 species of edible insects recognized in Korea, including locusts and edible silkworms (larvae and pupae), and bee drone pupae have been recognized as food ingredients in 2020.

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Recently, there has been a large-scale death of wintering bee colonies across the country, resulting to consequential damage and economic loss in beekeeping farms in Korea (Kim, 2022). Such events can be attributed to the aging of black locust (*Robinia pseudoacacia* L.) which is a major pollen source species, the decreased number of pollen sources, disease, as well as climate change. And as bees disappeared, honey production naturally decreased, which led to unstable income for beekeepers, making them look for new sources of income aside from honey production. In response to these challenges, drone pupae are being promoted to get recognized as a food raw material together with other edible insects as it could help improve the income of beekeepers. However, there is still more work needed to resolve negative perceptions of consumers towards edible insects, such as dislike towards the image of insects and safety concerns surrounding insects as food (Kim, 2018; Orsi *et al*., 2019).

Unlike worker bees, drone bees do not produce beekeeping products such as propolis and honey and consume only food without a role other than for procreation purposes. Therefore, drone pupae are discarded in beekeeping farms, except for mating (Kim *et al*., 2018). Drone pupae are high in protein and suitable for serving as dry food sources (Krell, 1996). In China, there is a custom of eating drone pupae and their pharmacological values have been described in Chinese medical books (Choi *et al*., 2009). Drone pupae have been assessed to have sufficient value as a food raw material in terms of food and nutrition (Ghosh *et al*., 2020; Gravel and Doyen, 2020) and has also been reported as potential source of various functional compounds with different bioactivities like anti-inflammatory (Kim *et al*., 2019; Ghosh *et al*., 2020), antioxidant (Ghosh *et al*., 2020; Kim *et al*., 2020) and antihyperglycemic (Kim *et al*., 2020), platelet-aggregating and antidiabetic (Pyo *et al*., 2020) effects. Thus, it is expected that drone pupae utilization as a food ingredient will increase significantly in the future. However, no research has been reported on chemical hazards that may occur during the production of drone pupae as well as the methods for managing the said hazards.

This study was conducted to find a suitable management method that can control and administer safe drone pupae production in beekeeping farms by analyzing chemical hazards such as heavy metals and pesticides that can occur in different stages of production. Through this, we would like to determine and propose critical limits for safe drone pupae production. To this end, beeswax (a breeding environment for drone pupae), domestic and imported pollen (which are raw and subsidiary materials) were analyzed together with drone pupae for presence of mycotoxins, animal drugs, pesticide residues, and heavy metals.

MATERIALS AND METHODS

1. Materials and reagents

The samples used in this study were collected from Jeollabukdo (Wanju and Jeongeup), Gyeonggido (Siheung), and Gyeongsangbukdo (Gyeongju) provinces of South Korea in May to June 2022. Drone pupae (*Apis mellifera* L.), beeswax, and imported pollen were collected from domestic beekeeping farms while domestic pollen was purchased online from beekeeping farms. The collected samples were kept and transported in an ice box with ice packs and stored at −80°C upon arrival at the laboratory prior to analysis.

Thermo Scientific™ QuEChERS Extraction Kit was purchased from ThermoFisher Scientific (Waltam, MA, USA). Aflatoxin Mixture (B1, B2, G1, and G2), ochratoxin A, T-2 toxin were obtained from Sigma-Aldrich (St. Louis, MO, USA) and fumonisin (B1, B2), deoxynivalenol, and zearalenone were purchased from Dr. Ehrenstorfer (Augsburg, Germany). HT-2 toxin was purchased from Romer Labs (Tulln, Austria). Nitric acid used in the heavy metals analysis was a special grade product (PFP, Japan). The mercury standard solution was prepared by diluting with 0.001% L-cysteine (98%, Nacalai Tesque Inc., Japan). To prepare calibration curves, standard solutions of lead, arsenic, and cadmium (1,000 mg/kg) were diluted with 5% nitric acid to prepare concentrations of 0.5–100 mg/kg. For mercury, the standard solution (1,000 mg/kg) was diluted with a 0.001% L-cysteine solution to prepare concentrations of 0.5–20 mg/kg for the calibration curves. Formic acid, ammonium formate, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), magnesium sulfate, sodium chloride, disodium citrate, trisodium citrate, nitric acid and acetone were purchased from Sigma-Aldrich (St. Louis, MO, USA)

and LC-MS grade acetonitrile and methanol were purchased from Merck Inc. (Darmstadt, Germany). All other reagents used in this study were of HPLC- or analytical-grade.

2. Methods

1) Chemical hazard analysis

(1) Mycotoxin

Samples were prepared following the methods of Jo *et al.* (2021) with slight modifications, 5 g of the homogenized sample and 10 mL of acetonitrile (ACN) containing 10% formic acid were placed in a 50 mL centrifuge tube and shaken for 30 minutes. Then, after adding 4 g $MgSO_4$ and 1 g NaCl, the mixture was shaken with a dedicated QuEChERS homogenizer (Thermo Scientific™ QuEChERS Extraction Kit, ThermoFisher Scientific, MA, USA) for 1 minute. Extraction was performed by centrifugation at 3,000 rpm/min for 10 minutes. The samples were further purified according to Seo *et al.* (2021), 1 mL of the supernatant was placed in a dispersive SPE tube containing 25 mg Primary Secondary Amine (PSA) and 25 mg C18, powder form, shaken for 1 minute, centrifuged at 10,000 rpm/min for 5 minutes, and filtered through a 0.2 μm syringe filter to be used as test solution. Finally, 400 μL of the test solution, 500 μL of distilled water, and 100 μL of acetonitrile were mixed to make up the final 1 mL for analysis.

Derivatized samples were tested for presence of aflatoxin (B1, B2, G1, G2), fumonisin (B1, B2), deoxynivalenol, ochratoxin A, zeralenon (α-zeralenol, β-zeralenol), T-2 toxin, and HT-2 using LC-MS/MS method (Seo *et al.*, 2021). A total of 12 mycotoxins were analyzed using 1290 Infinity II Liquid Chromatograph (Agilent, USA) and 6470 LC/TQ (Agilent, USA), with Poroshell 120 SB (150 mm × I.D 2.7 mm, 3.0 μm) as column. The mobile phases were 5 mM aqueous solution of ammonium formate (solvent A) and methanol solution containing 5 mM ammonium formate (solvent B), both of which contained 0.1% (v/v) formic acid. The samples were run at 0.3 mL/min flowrate through the following concentration gradient conditions: starting with 95% of solvent A, and solvent B was increased to 30% over 3 minutes, 60% within 5 minutes, 80% within 7 minutes, and 95% within 9 minutes, and then remained constant until 10 minutes. Solvent B was then decreased by 5% over 0.1 min and remained the same for 5.9 min. For LC, a 6470 LC/TQ (Agilent, USA) equipped with AJS Electrospray Ionization in positive ion mode was used. The analysis was performed by setting the gas temperature to 300°C, the gas flow to 7 L/min, the atomizer to 35 psi, the sheath gas temperature to 350°C, and the sheath gas flow to 11 L/min.

(2) Veterinary drugs

Veterinary drugs were analyzed following method stated in the Food Code (MFDS, 2021). 2 g of the homogenized sample was placed in a 50 mL centrifuge tube, and 5 mL of 10% aqueous EDTA solution was added and shaken for 1 minute. Thereafter, 5 mL of acetonitrile (ACN) was added and centrifuged at 3,000 rpm/min for 10 minutes. Then, 10 mL of ACN was added to the supernatant, followed by centrifugation at 3,000 rpm/min for 10 minutes. The resulting supernatant was added with 1 g of C18 powder and 10 mL of acetonitrile-saturated nucleic acid, shaken for 1 minute, centrifuged at 3,000 rpm/min for 10 minutes, and the upper layer supernatant was removed. Excluding the powder, 5 mL of the lower liquid layer was transferred to a new centrifuge tube and dried with nitrogen. The residue was dissolved in 1 mL of a mixed solution of water:methanol (1 : 1, v/v) and filtered through a 0.2 μm syringe filter to be used as a test solution.

As for the components to be analyzed, 92 types of veterinary drugs (Table 1) for livestock and aquatic products in the Food Code were selected and analyzed by simultaneous multi-component test method (MFDS, 2022). 1290 Infinity II Liquid Chromatograph (Agilent, USA) was used for LC-MS/MS analysis, using Eclipse Plus C18 RRHD (100 mm × I.D. 2.1 mm, 1.8 μm) as column. Two mobile phases, 5 mM aqueous solution of ammonium formate (solvent A) and 5 mM ammonium formate in methanol solution (solvent B) were used, with both solvents containing 0.1% (v/v) formic acid. The derivatized samples were run at 0.3 mL/min flowrate through concentration gradient conditions: starting with 95% of solvent A, and solvent B was increased to 30% from 1.5 to 3 minutes, 60% from 6 minutes, 90% from 10 minutes, and 98% from 12 minutes and kept constant until 13 minutes, solvent B was reduced by 5% over 0.1 min and held the same for 3.9 min. MS/MS conditions were established by setting the gas temperature to 250°C,

Table 1. List of compounds analyzed using LC-MS/MS according to MFDS Veterinary Drugs Multi-Component Analysis Method for Livestock and Other Aquatic Products

Acetanilide	DL-methylephedrine	Olaquindox_AG	Sulfaguanidine
Acriflavine	Doxycycline	Oleandomycin	Sulfamerazine
Altrenogest	Emamectinm	Orbifloxacin	Sulfamethazine
Aminopyrine	Enrofloxacin	Ormetoprim	Sulfamethoxazole
Ampicillin	Erythromycin	Oxacillin	Sulfamethoxypyridazine
Antipyrine	Florfenicol	Oxolinic acid	Sulfamonomethoxine
Benzylpenicillin	Florfenicol	Pefloxacin	Sulfaphenazole
Berberine	Flumequine	Phenacetin	Sulfaquinoxaline
Carbadox	Josamycin	Phenazone	Sulfathiazole
Cefalonium	Lincomycin	Phenothiazine	Sulfisoxazole
Chloramphenicol	Loperamide	Praziquantel	Tetramethrin
Chlortetracycline	Marbofloxacin	Ractopamine_AG	Tetramisole
Ciprofloxacin	Methomyl	Rifaximin_AG	Thiamphenicol
Clenbuterol	Metoclopramide	Ronidazole	Tiamulin
Cloxacillin	Metronidazole	Roxithromycin	Tildipirosin
Cyproheptadine	Monoacetyl dapson	Sarafloxacin	Tilmicosin
Danofloxacin	Nafcillin	Scopolamine	Trimethoprim
Dapsone	Nalidixic acid	Spiramycin	Tripelennamine
Desacetyl cephapirin	Naloxone	Sulfachloropyridazine	Tulathromycin
Dicloxacillin	Nandrolone	Sulfaclozine	Tylosin
Diethylcarbamazine	Norfloxacin	Sulfadiazine	Valnemulin
Difloxacin	Novobiocin	Sulfadimethoxine	Virginiamycin M1
Diphenhydramine	Ofloxacin	Sulfadoxine	Yohimbine

the gas flow to 10 L/min, the atomizer to 40 psi, the sheath gas temperature to 350°C, and the sheath gas flow to 12 L/min in the ESI positive mode.

(3) Pesticide residues

Pesticide residues were analyzed based on the Multi-component Analysis Method for Harmful Substances in Agricultural Products (MFDS, 2020). 10 g of the homogenized sample was placed in a 50 mL centrifuge tube, and 10 mL of ACN was added and shaken for 1 minute. Then, 4 g of anhydrous magnesium sulfate, 1 g of sodium chloride, 0.5 g of disodium citrate 1.5 hydrate, and 1.5 g of trisodium citrate 2 hydrate were added and shaken with a QuEChERS homogenizer (Thermo Scientific™ QuEChERS Extraction Kit, ThermoFisher Scientific, MA, USA) for 1 minute. After shaking, the mixture was centrifuged at 3,000 rpm/min for 5 minutes, and the supernatant was filtered through a 0.2 μm syringe filter to be used as a test solution. A simultaneous LC-MS/MS (207 types) and GC-MS/MS (113 types) multi-component analysis method for harmful substances in agricultural products (MFDS, 2020) were followed to check pesticide residues in samples using Shimadzu GC-2010 (Shimadzu, Japan) and Shimadzu TQ-8040 (Shimadzu, Japan). For GC-MS/MS, the column used for analysis was Rxi®-5Sil MS (20 mm × I.D 0.18 mm, 0.18 μm), sample injection amount 1 μL, inlet temperature 280°C, helium (99.9999%) was used as the carrier gas, and the flow rate was 1.5 mL/min. The initial temperature of the column was 50°C, maintained at this temperature for 1 minute, raised to 200°C at 25°C/min, and then raised to 300°C at 10°C/min and held for 5 minutes. The mass spectrometer used electron ionization (EI) positive ion (+) mode, electron energy was 70 eV, and argon (Ar) was used as the collision gas.

For LC-MS/MS analysis, Nexera X2 Liquid chromatograph (Shimadzu, Japan) and LC-MS-8050 (Shimadzu, Japan) were used. The column used was Phenomenex Kinetex C18 (150 mm × I.D 2.1 mm, 2.6 μm), the column temperature was 40°C, and the mobile phase was a 5 mM aqueous solution of ammonium formate (solvent A) and a methanol solution containing 5 mM ammonium formate (solvent B). All solvents contained 0.1% (v/v)

Table 2. List of pesticides analyzed using GC-MS/MS according to MFDS Pesticide Residue Multi-Component Analysis Method

GC-MS/MS			
Acrinathrin (2 isomers)	Dicofol	Heptachlor	Prochloraz
Alachlor	Dieldrin	Heptachlor-epoxide	Procymidone
Aldrin	Difenoconazole (2 isomers)	Imibenconazole	Promecarb
Ametoctradin	Dimethoate	Indanofan	Prometryn
Anilofos	Dimethylvinphos	Indoxacarb	Propachlor
Azaconazole	Diphenylamine	Iprodione	Propazine
Benfuresate	Disulfoton	Isazofos	Propiconazole (2 isomers)
BHC (alpha,beta,delta)	Endosulfan (alpha)	Isofenphos	Propisochlor
Bifenox	Endosulfan (beta)	Mecarbam	Propyzamide
Bifenthrin	Endosulfan-sulfate	Methidathion	Prothiofos
Bromobutide	Endrin	Metolachlor	Pyridalyl
Bromopropylate	EPN	Metribuzin	Quintozene
Butachlor	Epoxiconazole	Lindane (gamma-BHC)	Silafluofen
Butafenacil	Ethalfluralin	Oxyfluorfen	Simazine
Carbophenothion	Ethion	o,p–DDT	Simeconazole
Chlorantraniliprole	Etridiazole	p,p–DDD	Simetryn
Chlordane (2 isomers)	Fenclorim	p,p–DDE	Spiromesifen
Chlorfenapyr	Fenitrothion	p,p–DDT	Tebupirimfos
Chlorfenvinphos (2 isomers)	Fenothiocarb	Parathion-ethyl	Tefluthrin
Chlorfluazuron	Fenoxanil	Parathion-methyl	Terbufos
Chlorobenzilate	Fenpropathrin	Pendimethalin	Terbutryn
Chlorpropham	Fenthion	Penthiopyrad	Tetradifon
Chlorpyrifos-methyl	Fenvalerate (2 isomers)	Permethrin (2 isomers)	Thifluzamide
Cyfluthrin (4isomers)	Fipronil	Phenothrin (2 isomers)	Tolclofos-methyl (TPP)
Cyhalothrin	Flucythrinate (2 isomers)	Phorate	Triadimenol
Cypermethrin (4 isomers)	Flumioxazine	Phosalone	Tri-allate
Cyprodinil	Fluopyram	Picoxystrobin	Trifluralin
Deltamethrin (tralomethrin)	Fonofos	Piperonyl butoxide	Vinclozolin
Diclofop-methyl	Fthalide	Pirimiphos-ethyl	Zoxamide
Dicloran	Halfenprox	Pretilachlor	

formic acid. The concentration gradient conditions were started with 95% of solvent A, and solvent B was increased to 60% for 1.5–2.4 minutes and 90% for 10 minutes, and then kept constant for 2 minutes. After that, it was kept constant at 98% from 12.1 to 18 minutes, then decreased to 5% at 18.1 minutes, and then kept constant until 24 minutes. The pesticide residues analyzed were shown in Tables 2 and 3.

(4) Heavy metals

The samples were analyzed for presence of lead, cadmium, and mercury according to the method in the Food Code (MFDS, 2021). Sample pretreatment for lead and cadmium analysis was performed using the microwave method. After taking 1 g of the homogenized sample in a microwaveable vessel, 7 mL of nitric acid and 1 mL of hydrogen peroxide were added. After pre-oxidation at 80°C for 15 minutes in a heating block, microwave digestion system was used to release electromagnetic waves for the first 5 minutes to raise the temperature from room temperature to 120°C, and after maintaining 120°C for a total of 5 minutes excluding electromagnetic waves, 1 minute electromagnetic waves were further emitted and degraded under electromagnetic waves for 1 minute or until conditions of full degradation. After the decomposition was completed, the acid decomposition product was put in a 20 mL volume flask, diluted with triple distilled water, and the mixture passed through Filter Paper (5B, 110 mm) and was used as the test solution.

Table 3. List of pesticides analyzed using LC-MS/MS according to MFDS Pesticide Residue Multi-Component Analysis Method

LC-MS/MS

Acephate	Daimuron	Isoprothiolane	Propanil
Acetamiprid	Edifenphos	Isopyrazam	Propaquizafop
Aldicarb	Esprocarb	Kresoxim-methyl	Propoxur
Amisulbrom	Ethaboxam	Linuron	Pyraclofos
Azimsulfuron	Ethiofencarb	Lufenuron	Pyraclostrobin
Azinphos-methyl	Etofenprox	Malathion	Pyrazolate
Azoxystrobin	Ethoprophos	Mandipropamid	Pyrazophos
Bamectin B1	Ethoxysulfuron	Mefenacet	Pyribenzoxim
Bendiocarb	Etoxazole	Mepanipyrim	Pyributicarb
Bensulfuron-methyl	Etrimfos	Mepanipyrim	Pyridaben
Benthiavalicarb-isopropyl	Famoxadone	Mepronil	Pyridaphenthion
Benzobicyclon	Fenamiphos	Metalaxyl	Pyrifluquinazon
Benzoximate	Fenarimol	Metamifop	Pyriftalid
Bitertanol	Fenazaquin	Metazosulfuron	Pyrimethanil
Boscalid	Fenbuconazole	Metconazole	Pyrimidifen
Bromacil	Fenhexamid	Methabenzthiazuron	Pyriminobac-methyl (E,Z)
Buprofezin	Fenobucarb	Methiocarb	Pyrimisulfan
Cadusafos	Fenoxaprop-ethyl	Methomyl	Pyriproxyfen
Cafenstrole	Fenoxycarb	Methoxyfenozide	Pyroquilon
Carbaryl	Fenpyroximate	Metobromuron	Quinalphos
Carbendazim	Fentrazamide	Metolcarb	Quinmerac
Carbofuran	Ferimzone (E,Z)	Metrafenone	Quinoclamine
Carboxin	Flonicamid	Mevinphos	Quizalofop-ethyl
Carfentrazone-ethyl	Fluacrypyrim	Milbemectin A3	Saflufenacil
Carpropamide	Flubendiamide	Milbemectin A4	Sethoxydim
Chlorpyrifos	Flucetosulfuron	Molinate	Spinetoram (J,L)
Chlorsulfuron	Fludioxonil	Monocrotophos	Spirodiclofen
Chromafenozide	Flufenacet	Myclobutanil	Spirotetramat
Clethodim	Flufenoxuron	Napropamide	Sulfoxaflor
Clofentezine	Fluopicolide	Nicosulfuron	Tebuconazole
Clomazone	Fluxapyroxad	Novaluron	Tebufenozide
Clothianidin	Fluquinconazole	Nuarimol	Tebufenpyrad
Cyazofamid	Flusilazole	Ofurace	Terbuthylazine
Cyclosulfamurom	Flutolanil	Omethoate	Tetraconaole
Cyflufenamid	Forchlorfenuron	Oxadiazon	Thenylchlor
Cyhalofop-butyl	Fosthiazate	Oxadixyl	Thiabendazole
Cymoxanil	Furathiocarb	Oxamyl	Thiacloprid
Cyproconazole (I,II)	Gibberellic acid	Oxaziclomefone	Thiamethoxam
Dichlovos (DDVP)	Halosulfuron-methyl	Paclobutrazole	Thiazopyr
Demeton-S-Methyl	Haloxyfop	Penconazole	Thidiazuron
Diazinon	Hexaconazole	Pencycuron	Thifensulfuron-methyl
Diethofencarb	Hexaflumuron	Penoxsulam	Thiobencarb
Diflubenzuron	Hexazinone	Pentoxazone	Thiodicarb
Dimepiperate	Hexythiazox	Phenthoate	Tiadinil
Dimethametryn	Imazalil	Phosphamidone	Triadimefon
Dimethenamid	Imazosulfuron	Phoxim	Triazophos
Dimethomorph (E,Z)	Imicyafos	Piperophos	Tricyclazole
Diniconazole	Imidacloprid	Pirimicarb	Trifloxystrobin
Dinotefuran	Inabenfide	Pirimiphos-methyl	Triflumizole
Diphenamid	Iprobenfos	Probenazole	Triflumuron
Dithiopyr	Iprovalicarb	Profenofos	Uniconazole
Diuron	Isoprocarb	Propamocarb	

The presence of lead and cadmium analysis were investigated using ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry), and the equipment used in this experiment was 5110 ICP-OES (Agilent, USA). For the analysis conditions, the gas flow rates were plasma flow 12.0 L/min, nebulizer flow 0.65 L/min, and Aux flow 1.00 L/min, and analyzed with RF power 1.45 kw.

Sample pretreatment for the analysis of mercury was done by the nitric acid digestion method; 0.5 g of the sample and 3 mL of nitric acid were put in a 20 mL flask and heated at 80°C for 30 minutes in a heating block. After, digested sample was diluted with triple distilled water, filtered (5B filter paper, 110 mm), and used as a test solution. At the time of analysis, derivatized sample filtrates were diluted 5 to 10 times to prepare a final 100 mL test solution.

Mercury analysis was analyzed using atomic absorption spectrometry (AAS), and after adding 5 mL of sulfuric acid and 5 mL of saturated stannous chloride solution to 100 mL of the test solution, it was analyzed by installing a mercury vapor generator. SHIMADZU AA-62200 spectrophotometer (Shimadzu, Japan) was used, and analysis conditions were mercury wavelength of 253.7 nm, lamp current of 4 mA, and slit width of 0.7 nm.

(5) Limits of detection (LOD) and quantification (LOQ)

The calibration curve slope and the standard deviation of the peak area of the lowest concentration in the calibration curve were determined using the following equations to calculate the limit of detection (LOD) (1) and limit of quantitation (LOQ) (2):

$$LOD = 3.3 \times \sigma/S \ (S/N = 3) \quad (1)$$

$$LOQ = 10 \times \sigma/S \ (S/N = 10) \quad (2)$$

where σ is the average standard deviation of the peak area at the lowest concentration and S is the average slope of the calibration curve.

(6) Hazard assessment of the pesticide residues

The hazard assessment of detected pesticides was conducted by calculating the estimated daily intake (EDI), which was derived by multiplying the average residue level (mg/kg) of the detected pesticide by the daily consumption (g/day) of each sample. The daily consumption was assumed to be 3 g, which is the standard serving size of commercially available insect-processed foods. The maximum permissible intake (MPI, mg/person/day) of each pesticide was calculated by multiplying the acceptable daily intake (ADI, mg/kg b.w./day) provided by the database of the Ministry of Food and Drug Safety by the average body weight of Koreans, which is 66.55 kg (KOSIS, 2021).

RESULTS

1. Method validation

Suitability of the proposed method for determining mycotoxins, pesticides, veterinary drugs, and heavy metals were validated according to the guidelines in SANTE/126823/2019 (EC, 2019), MFDS manual (2020). Table 4 summarizes the recovery, LOD and LOQ for mycotoxins, pesticides, veterinary drugs, and heavy metals in the samples tested. The recovery rate of mycotoxins ranged from 74.0% to 99.8%, and the limits of detection (LOD) and quantification (LOQ) were 0.05–1.63 mg/kg and 0.18–6.34 mg/kg, respectively. For pesticides, the recovery rate was 91.6–103.8%, and the LOD and LOQ values were 0.0005 mg/kg and 0.001 mg/kg, respectively. The recovery rate of veterinary drugs was 63.2–110.9%, and the LOD and LOQ were both 0.0005 mg/kg and 0.001 mg/kg, respectively. For heavy metals, the recovery rate was 92.3–102.0%, and the LOD and LOQ were 0.11–2.02 mg/kg and 0.34–6.12 mg/kg, respectively.

2. Mycotoxin analysis

The samples were analyzed for presence of aflatoxin (B1, B2, G1, G2), Fumonisin (B1, B2), Deoxynivalenol, Ochratoxin A, Zeralenon (α-zeralenol, β-zeralenol), T-2 toxin, and HT-2 toxin, a total of 12 mycotoxins, and no mycotoxins were detected in all samples (Table 5).

3. Veterinary drugs analysis

The samples were tested for 92 types of veterinary drugs and no residual veterinary drugs were detected in drone pupae and beeswax. Berberine was found in domestic pollen and trimethoprim in imported pollen at less than 0.007 mg/kg each (Table 6).

Table 4. Recovery, LOD, and LOQ for mycotoxin, pesticide, veterinary drugs, and heavy metals

Compounds		Conc. (mg/kg)	Recovery (%)	LOD (mg/kg)	LOQ (mg/kg)
Mycotoxins	B1	5	77.6	0.18	0.59
	B2	5	78.0	0.10	0.34
	G1	1.25	74.0	0.08	0.26
	G2	1.25	85.3	0.05	0.18
	FB1	200	89.6	0.88	2.89
	FB2	200	79.6	1.92	6.34
	OTA	10	83.5	0.11	0.38
	DON	500	79.2	1.63	5.37
	T2	25	80.3	0.09	0.31
	HT-2	25	89.2	0.68	2.23
	ZEN(α)	200	94.1	0.12	0.38
	ZEN(β)	200	99.8	0.10	0.31
Pesticides	Azoxystrobin	1	98.9	0.0005	0.007
	Chlorpyrifos	1	91.6	0.0005	0.007
	Diazinon	1	103.8	0.0005	0.007
Veterinary Drugs	Berberine	10	63.2	0.0005	0.007
	Trimethoprim	10	110.9	0.0005	0.007
Heavy Metals	Pb	100	97.8	1.54	4.70
	Cd	100	92.3	0.11	0.34
	Hg	100	102.0	0.78	2.35

Table 5. The contents of mycotoxin in drone pupas, beehive, domestic bee pollen, and imported bee pollen

Sample	Detection	No. of detection/ Sample No. (%)
Drone pupae	ND[a]	0/10 (−)
Beehive	ND	0/10 (−)
Bee pollen (Domestic)	ND	0/10 (−)
Bee pollen (Imported)	ND	0/10 (−)

[a]ND: Not detected (below the detection limit of analysis)

4. Pesticide residue analysis

Table 7 shows the results of analyzing 113 pesticide residues by GC-MS/MS and 207 pesticide residues by LC-MS/MS. In drone pupae, pesticide residues were not detected in all samples. Consequently, pesticide residues were detected in 40% of beeswax, 30% of domestic pollen, and all samples of imported pollen. Residual pesticides detected in beeswax were fludixonil, trifloxystrobin, cyprodinil, and penthiopyrad. Trifloxystrobin was found highest with 4 cases, and cyprodinil was noted at 0.078 mg/kg. In domestic pollen, azoxystrobin, chlorpyrifos, and diazinon were detected at less than 0.007 mg/kg, for each pesticide. Residual pesticides found in imported pollen were carbendazim, haloxyfop, and triadimenol, with haloxyfop being the highest amount at 0.267 mg/kg.

5. Heavy metal analysis

Heavy metals were detected in drone pupae, beeswax, and domestic and imported pollen samples (Table 8). Mercury was detected at less than 0.001 mg/kg in all samples. Lead and cadmium were detected with the highest concentrations in domestic pollen at 0.629 mg/kg and 0.222 mg/kg, respectively.

6. Hazard assessment of the detected pesticide residues

The results of the hazard assessment (Table 9) of the detected pesticides showed that the %ADI (Hazard index) ranged from 9.8 to 780.0% in beehive samples, indicating a very high risk if the pesticides were transferred to the drone pupae from the beehive. In imported bee

Table 6. Veterinary drugs in drone pupae, beehive, domestic bee pollen, and imported bee pollen

Sample	Detection	No. of detection/ Sample No. (%)	Detection level (mg/kg)
Drone pupae	ND[a]	0/10 (−)	−
Beehive	ND	0/10 (−)	−
Bee pollen (Domestic)	Berberine	10/10 (100)	<0.007
Bee pollen (Imported)	Trimethoprim	10/10 (100)	<0.007

[a]ND: Not detected (below the detection limit of analysis)

Table 7. Pesticide residues in drone pupae, beehive, domestic bee pollen, and imported bee pollen

Sample	Detection	No. of detection/ Sample No. (%)	Detection level (mg/kg)
Drone pupae	ND[a]	0/10 (−)	−
Beehive	Fludioxonil	3/10 (30)	0.013±0.001
	Trifloxystrobin	4/10 (40)	<0.007
	Cyprodinil	2/10 (20)	0.078±0.011
	Penthiopyrad	2/10 (20)	0.012±0.006
Bee pollen (Domestic)	Azoxystrobin	2/10 (20)	<0.007
	Chlorpyrifos	1/10 (10)	<0.007
	Diazinon	2/10 (20)	<0.007
Bee pollen (Imported)	Carbendazim	10/10 (100)	<0.007
	Haloxyfop	10/10 (100)	0.267±0.019
	Triadimenol	10/10 (100)	0.048±0.004

[a]ND: Not detected (below the detection limit of analysis)

Table 8. Heavy metal contents (Pb, Cd and Hg) in drone pupae, beehive, domestic bee pollen, and imported bee pollen

Sample	Detection	No. of detection/ Sample No. (%)	Detection level (mg/kg)
Drone pupae	Pb	10/10 (100)	<0.2
	Cd	10/10 (100)	<0.1
	Hg	10/10 (100)	<0.001
Beehive	Pb	10/10 (100)	0.212±0.027
	Cd	10/10 (100)	<0.1
	Hg	10/10 (100)	<0.001
Bee pollen (Domestic)	Pb	10/10 (100)	0.629±0.333
	Cd	10/10 (100)	0.222±0.174
	Hg	10/10 (100)	<0.001
Bee pollen (Imported)	Pb	10/10 (100)	<0.2
	Cd	10/10 (100)	<0.1
	Hg	10/10 (100)	<0.001

Table 9. Hazard assessment for pesticide detected in beehive and imported bee pollen

Sample	Pesticide	Average con. (mg/kg)	Food daily intake (g/day)	ADI	EDI	MPI	%ADI	%MPI
Beehive	Fludioxonil	0.013	3	0.40000	0.039	26.620	9.8	0.1465
	Cyprodinil	0.078	3	0.03000	0.234	1.997	780.0	11.7205
	Penthiopyrad	0.012	3	0.08100	0.036	5.391	44.4	0.6678
Bee pollen (Imported)	Haloxyfop	0.267	3	0.00065	0.801	0.043	123230.8	1851.7020
	Triadimenol	0.048	3	0.03000	0.144	1.997	480.0	7.2126

ADI (Acceptable daily intake, mg/kg b.w./day)
EDI (Estimated daily intake, mg/kg b.w./day) = concentration of detection (mg/kg) × daily food intake (g/day)/(body weight)
MPI (Maximum permissible intake, mg/man/day) = ADI × 66.55 kg (average body weight)
%ADI (%Acceptable daily intake; Hazard index) = (EDI/ADI) × 100
%MPI (%Maximum permissible intake) = (EDI/MPI) × 100

pollen, the %ADI was as high as 123,230%. Although pesticide residues can be partially removed through washing and processing, safety management is still needed for drone pupae and their rearing environment to ensure safety.

DISCUSSION

1. Chemical hazard management plan for safe drone pupae production

Mycotoxins are secondary metabolites of fungi, and major toxins include aflatoxin, fumonisin, ochratoxin, and zeralenone. The factors that cause mycotoxins are influenced by the environment, storage conditions, and ecological conditions. Mycotoxins cause complex economic damage, such as loss of human life, loss of livestock production, loss of feed control and intensive research funds (Lee *et al*., 2002). In addition, some mycotoxins not only directly cause disease when ingested by livestock, but may also remain in the tissue of livestock food, which may cause secondary harm to humans when these foods are consumed (NIFDS, 2014).

The fact that mycotoxins were not detected in all samples of male bee pupae, beeswax, domestic pollen and imported pollen seems to be because bees use propolis, which has a bactericidal and preservative effect to make the inside of the beeswax aseptic and prevent the invasion of harmful microorganisms (Ghisalberti, 1979).

To prevent mycotoxin, pollen (feed) must be purchased from an approved supplier, and upon receipt, a thorough sensory test must be performed to confirm whether mold has occurred or not. It is also good to check the product inspection report if necessary. In addition, when storing the produced drone pupae, it is important to pay attention to the temperature and humidity control to prevent mold from occurring and to keep the storage area clean.

Veterinary drugs are classified into antibiotics, vaccines, hormones, etc., and are used for the treatment and prevention of diseases in livestock and aquatic products. Since they can remain in food, management of veterinary drugs is necessary to ensure food safety. Residual substances refer to small amounts of substances left in food due to intentional use, such as veterinary drugs and pesticides (CWNU, 2021). If the veterinary drug residues are exposed to the human body as food for a long time (accumulated exposure), there is a possibility of harm to humans, the final consumer, which could lead to resistance to human drugs (Oh *et al*., 2009).

Berberine detected in domestic pollen is used as an antidiarrheal agent for cattle, pigs, and horses while trimethoprim detected in imported pollen is used as an antibacterial agent for various species such as cattle, pigs, deer, and fish. Although both components are not included in the residual standards for honey in the Food Code, they were at acceptable levels in the residual standards for other livestock species. The limit for berberine is 0.01 mg/kg and for trimethoprim is considered safe at 0.02−0.1 mg/kg (MFDS, 2022). However, it seems necessary to investigate the use of chemicals in the farmhouse and the environment around the farm to identify the route of contamination with ingredients other than those allowed for major diseases of bees.

As the demand for strengthening regulations on residual substances intensifies to prevent misuse and abuse of veterinary drugs and strengthen livestock product safety management, the PLS (Positive List System) for livestock and aquatic veterinary drugs will be introduced from January 2024. Against this background, care must be taken to ensure that no veterinary drugs remain in the production of drone pupae. To prevent the trace levels of veterinary medicines, when bringing in pollen (feed), pollen must be purchased from an approved supplier. And when bringing in medicines, use instructions, dosage, withdrawal period, etc. must be checked through the instruction manual. In addition, it is good to periodically conduct a drug transaction ledger check and inventory survey. When using drugs in the breeding stage, the proper concentration used should be strictly observed, and treatment details should be recorded and managed in the drug consumption record book. After collection, a residual substance test should be conducted to separate and dispose of pupae that are suspected of with high presence or with amount remaining above the permissible level.

Pesticide residues refer to trace amounts of pesticides that remain in agricultural products after pesticide use (MFDS, 2015). The types of pesticides are very diverse, and the types and amounts used vary depending on crops and agricultural environment conditions. Pesticide residues in food belong to important hazardous chemicals that require a high level of management technology, and systematic safety regulation methods are required. Therefore, the maximum residue limit (MRL) is legally established and managed (Lee, 2010).

Fludixonil, trifloxystrobin, cyprodinil, and penthiopyrad detected in beeswax, azoxystrobn, chlorpyrifos, and diazinon detected in domestic pollen, and carbendazim, haloxyfop, and triadimenol detected in imported pollen all met the residue standard limits for agricultural and livestock products in the Food Code. However, cyprodinil detected in beeswax was not specified as a honey pesticide residue standard and even exceeded the residual tolerance standard of 0.01 mg/kg for other livestock products. Therefore, it is considered necessary to manage pesticide residues in drone pupae without spraying insecticides containing pesticide chemicals at the time of collecting drone pupae, and to also prevent pesticides from entering the nearby environment. Additionally, it would be more valuable to check the inspection report when raw and subsidiary materials are received, and to monitor pesticide residues by conducting a residual substance test after collection.

When heavy metals enter the environment, they accumulate in the body of animals and plants through the food chain and finally congregate in the human body. It is not decomposed during food manufacturing and processing, and it is not degraded in the body, so it has high accumulation. A large amount of heavy metal exposure causes acute and chronic poisoning, so it is seriously related to food safety (MFDS, 2016).

Lead is used for various purposes in life, but it is a component that is not necessary for the structure and function of the human body. It is exposed to the human body through air, soil, groundwater, food, etc., and continuous and excessive exposure through direct or indirect routes affects the central nervous system, stomach, bloodstream, nervous system, pregnant woman and fetus, immune system, etc., and is also related to cancer (Lee, 1993).

Cadmium is found in many foods for human consumption due to its high mobility from soil to agricultural products. In Korea, most of the rivers are contaminated with cadmium due to dumping of industrial wastewater and waste ores, and agricultural products are highly likely to be exposed to cadmium when they are used as agricultural water. Exposure to cadmium is a major health burden, resulting in increased mortality from cancer, *itai-itai* disease, and interference with the reabsorption of nutrients and vitamins. In addition, the association with diseases such as diabetes, hypertension, and lung disease has been confirmed from epidemiological studies (Satarug, 2010).

Mercury is used as an essential metal for life and is widely used in medicine and industry. It exists in many environments, including air, water, and soil, and can move continuously to various environments. Mercury introduced into the ecosystem has a fatal effect and is known to have a wide range of hazards from fetuses to adults, as well as, carcinogenic effects (Kim, 2008). It causes central nervous system disorders mainly due to brain damage and causes more fatal damage to fetuses and children than adults (Kim *et al*., 2007).

For heavy metals analysis, lead, cadmium, and mercury were detected in drone pupae, beeswax, and dome-

stic and imported pollen samples, but all of them meet the standard limits, respectively. To prevent heavy metal contamination in the production of drone pupae, it is necessary to use products that meet the standards and specifications of utensils, containers, and packaging, and to receive periodic test reports and periodic water tank cleaning and maintenance is required.

CONCLUSION

The 40 samples were analyzed for presence of chemical hazards (7 mycotoxins, 92 veterinary drugs, 320 pesticide residues, and 3 heavy metals) at different stages of drone pupae production. No mycotoxins were present in all samples. Veterinary drugs such as berberine was found in domestic pollen and trimethoprim was noted in imported pollen at less than 0.007 mg/kg, which were within limits of the residue standards for livestock products but were not included in the residue standards of the Food Code for honey. Residual pesticides were detected in beeswax (fludixonil, trifloxystrobin, penthiopyrad), domestic pollen (azoxystrobn, chlorpyrifos, diazinon), and imported pollen (carbendazim), which were at within safe levels of residue standards for agricultural and livestock products in the Food Code. However, cyprodinil detected in beeswax was not included in the pesticide residue standard for honey and exceeded the residue limit of 0.01 mg/kg for other livestock products. Heavy metals were detected in all samples, but at trace levels meeting the criteria limits. Management of pesticides and veterinary medicines from the nearby environment should be strictly done to prevent chemical hazards from getting into drone pupae during production. Also, checking the inspection report when receiving raw and subsidiary materials and conducting a residual substance inspection after collection should also be properly implemented for safe drone pupae production.

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