Research Article

In-Cell Clean-Up Pressurised Liquid Extraction Method to Determine Pesticides in Mushroom Compost by Gas Chromatography-Tandem Mass Spectrometry

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A fast, simple, and easily automated method for the determination of two insecticides, diazinon and deltamethrin, and two fungicides, iprodione and prochloraz, in mushroom cultivation compost samples, based on selective pressurised liquid extraction (SPLE) and gas chromatography coupled to tandem mass spectrometry, is presented. The proposed method integrates extraction and clean-up processes in one single step, by adding a clean-up sorbent into the extraction cell. SPLE variables were thoroughly studied by experimental design. First, different clean-up sorbents and extraction solvents were screened at two temperature levels using a multifactor design; resulting Florisil and 1:1 acetone-dichloromethane the best combination. Then, temperature, extraction time, and sample-sorbent mass ratio were optimized by a central composite design. Best recoveries were obtained with a 0.4 sample-sorbent ratio, at 105°C and a 2 min extraction time. The SPLE method was characterized in terms of recovery (with values ranging from 81 to 103%), repeatability and intermediate precision (showing relative standard deviations less than 12% in most cases), and sensitivity (providing detection limits between 0.1 and 6 ng mL⁻¹). However, in spite of the clean-up process a matrix effect was observed and therefore standard addition calibration was recommended.

1. Introduction

Compost for mushroom growing is made from several agricultural byproducts (straw, chicken manure, etc.) which are fermented and pasteurised. Therefore, the final product is formed by a complex mixture of nutrients resulting from the organic matter decomposition. During mushroom growing, chemicals such as fungicides, insecticides, or pesticides are added to the crop to maintain the mushroom immune to harmful insects, weeds, or microbes. After growing, exhausted substrate can be reutilised as supplement for crops, soil conditioner, mulch, agent of plant disease suppression, and soil bioremediation [1, 2]. Therefore, the levels and toxicity of these chemicals must be controlled in order to avoid environmental pollution and human exposure to pesticides.

The origin, concentration, and degradation of several persistent organic pollutants such as polynuclear aromatic

hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins and -furans (PCDD/Fs) in compost have been studied [3] due to emerging concern about their impacts to the environment. Diazinon, iprodione, prochloraz, and deltamethrin (Figure 1) are pesticides commonly used in mushroom cultivation that are able to react as hormone agonists or antagonists [4].

Although there are many studies on the individual determination of these four target compounds in several matrices, a method for their simultaneous determination in compost has not been reported yet.

In recent years, many efforts have been made to determine pesticides in several samples such as fatty vegetable matrices [5], fruits [6], and food [7]. The analytical methods applied in laboratories for determining pesticides are usually time consuming, labour intensive, and complex; so in the last few years QuEChERS (quick, easy, cheap, effective,

FIGURE 1: Chemical structure of the target pesticides.

rugged, and safe) methodology, based on liquid partitioning combined with SPE, has been developed in order to achieve an effective extraction and cleanup approach [7].

The aim of this work is to develop a fast, simple, automated, and effective method for determining pesticides in compost using selective pressurised liquid extraction (SPLE). Pressurised liquid extraction (PLE) followed by a GPC or SPE clean-up step has been widely used for determining pesticides in vegetable matrices [8], in tobacco [9], in compost [10], and so forth. SPLE is a PLE process involving the addition of a sorbent in the extraction cell. In this approach, analytes are efficiently extracted from the sample at high pressure and temperature and simultaneously most of the coextracted compounds are retained in the sorbent, improving the extraction selectivity and providing a cleaner extract. Therefore, extraction and clean-up steps are integrated and a faster and simpler method is obtained [11]. This strategy has been successfully applied for determining parabens and triclosan in indoor dust [12], PBDEs in indoor dust [13], bisphenol A and alkylphenols in sewage sludge [14], and pesticides in seaweeds [15] but it has not been applied to pesticide extraction from compost yet.

In this work, a SPLE-based method for determining pesticides in compost from mushroom cultivation is developed and optimised. Several variables that affect the extraction step, such as solvent, sorbent, time, and temperature are studied and optimised by means of different experimental designs. Separation and determination of the pesticides is carried out by PTV-LV injection followed by GC-MS/MS. As a result, a fast, simple, sensitive, reliable, and robust method is accomplished.

2. Experimental

2.1. Chemicals, Materials, and Samples. Trace analysis grade solvents, such as methanol, ethyl acetate, acetone, and dichloromethane were acquired from Scharlab (Barcelona, Spain). Diazinon, iprodione, prochloraz, deltamethrin, and caffeine (internal standard) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). An individual stock standard solution of caffeine and a mixture stock standard solution of the four pesticides were prepared in ethyl acetate and stored at -18°C .

Anhydrous sodium sulphate was obtained from Scharlab (Barcelona, Spain). Florisil (60–100 mesh) was provided by Alfa Aesar (Karlsruhe, Germany), activated neutral aluminium oxide (150 mesh) and silica (150 mesh) were obtained from Sigma-Aldrich (Steinheim, Germany) and sea sand (50–70 mesh) was obtained from VWR-Prolabo (Mollet del Vallès, Barcelona, Spain). Florisil, alumina, and silica sorbents were dried at 140°C for 24 h and then allowed to cool down in a desiccator before use. Cellulose filters (20-mm diameter) for PLE were purchased by Restek (Bellefonte, PA, USA).

Compost samples from mushroom cultivations were provided by a mushroom research centre (CTICH, *Centro Tecnológico de Investigación del Champiñón de La Rioja*), and stored at -18°C before processing. They were triturated and chopped up with a domestic blender. Spiked samples were prepared by adding the four-pesticide mixture stock standard solution to the wet compost, and this mixture was homogenized in a mortar. The final concentration of each pesticide in the spiked compost was $1.6\text{ }\mu\text{g g}^{-1}$.

2.2. Selective Pressurised Liquid Extraction. Extractions were carried out using an ASE 200 pressurized liquid extractor from Dionex (Sunnyvale, CA, USA), furnished with 11 mL stainless-steel cells. Extraction cells were filled as follows. Two cellulose filters followed by 1 g of sodium sulphate were placed at the bottom of each cell. After loading the corresponding amount of clean-up sorbent, and a second layer consisting of a dispersion of sample, anhydrous Na_2SO_4 and sorbent was laid. The remaining cell void volume was filled with anhydrous sodium sulphate and another cellulose filter was placed on the top.

Different SPLE conditions were tested along the study of variables. Samples were processed under the following final optimised conditions. A compost sample amount of 1.0 g was dispersed in 2.0 g of anhydrous sodium sulphate and mixed with 0.5 g of Florisil using a mortar and a pestle. The mixture was then transferred to the extraction cells, over a layer of 2.0 g of Florisil. All extractions were performed at 1500 psi, at 105°C for 2 min. Extracts were evaporated under nitrogen stream until dryness using a Turbo Vap II concentrator (Zymark, Hopkinton, MA, USA) and then reconstituted with 2 mL of ethyl acetate. Before GC-MS/MS analysis, extracts were filtered through a 0.45 μm nylon filter, and caffeine was added as internal standard.

2.3. Gas Chromatography-Tandem Mass Spectrometry Analysis. Analyte separation was performed in a Varian CP 3800 gas chromatograph (Walnut Creek, CA, USA) coupled to a Varian Saturn 2200 ion trap MS/MS detector and furnished with a Varian Factor Four VF-5MS capillary column (30 m $\times$ 0.25 mm i.d., 0.25 μm of 5% polydiphenylsiloxane, 95% polydimethylsiloxane phase) and a CombiPal (CTC Analytics, Zwingen, Switzerland) autosampler. Helium (99.996% purity from Praxair España, Madrid, Spain) was employed as carrier gas with a constant flow of 1.0 mL min^{-1} . Large Volume Injections (LVIs) were carried out using a 1079 programmed-temperature vaporizer (PTV) injector, equipped with cryogenic CO_2 cooling. Injection volume was of 10 μL (using a 100- μL syringe) and a 3.4 mm internal diameter glass-frit-packed gooseneck insert was used. Initial injector temperature, 70°C, was held for 0.5 min and then increased until 300°C at a rate of 100°C min^{-1} , maintained at this value for 28.0 min. The split-splitless program consisted of four steps: the split vent valve was initially opened for 0.5 min at a split ratio of 1 : 100, after that it was closed for splitless injection for 3.0 min. Next, the valve was opened again for 26.50 min at a 1 : 100 split ratio to flush the injection liner and then the split ratio was changed to 1 : 20 to save carrier gas. Initial oven temperature was set at 70°C, held for 2.00 min, ramped at 10°C min^{-1} up to 300°C and held for 6 min, resulting in a total time of 31.00 min. Electron ionisation was used for iprodione, prochloraz and deltamethrin; whereas positive chemical ionisation for diazinon. Ions (m/z) and conditions selected for MS/MS detection are listed in Table 1. Excitation amplitude voltage optimisation for each pesticide was accomplished using the automated method development (AMD) tool included in the 6.9.1-version Varian MS Workstation software tool kit.

2.4. Software for Statistical Analysis. Experimental designs and statistical analysis were performed using Statgraphics Centurion XV (Statpoint, Herndon, VA, USA), and Microsoft Excel was used for drawing plots.

3. Results and Discussion

3.1. Sample Treatment. Compost samples presented high water content, around 70% (m/m), which should be reduced before PLE in order to avoid analyte partitioning in the aqueous phase. Three different drying approaches were tested: freeze-drying, oven heating at 60°C, and the use of anhydrous sodium sulphate as desiccant. The two first techniques produced analyte losses by volatilization or thermal degradation; therefore they were discarded. Sample and desiccant were mixed using a mortar and a pestle. A 1 : 2 sample-desiccant ratio was enough to obtain a dried-like soft-texture mixture.

3.2. Study of SPLE Variables. The study of the influence of SPLE variable was accomplished in several steps. First, extraction solvent and clean-up sorbent were screened at two temperature levels by means of a multicategorical factorial design. Once solvent and sorbent were selected, the influence of the main variables, temperature, time, and sample-sorbent mass ratio was studied with a central composite design and the response surface technique was used to determine the optimal values for these variables. Then, the effect of the sorbent distribution in the extraction cell was evaluated from one situation in which all the sorbent was mixed with the sample to another consisting of placing it in a layer over the sample bed. Finally, influence of the number of SPLE cycles on analyte recovery was studied.

3.2.1. Solvent and Sorbent Screening. In order to select the best solvent and sorbent, the effect of six solvents and three sorbents was studied at two temperature levels through a $6 \times 3 \times 2$ factorial design.

Ethyl acetate, acetone, and dichloromethane were selected as solvents because they are usually employed to extract the target analytes [8, 9, 16]. Moreover, their 1 : 1 binary mixtures were included in the study, and therefore a total of six levels were selected for the solvent factor.

Three Sorbents: Florisil, alumina, and silica, widely used to clean organic extracts and previously reported for SPLE [12–14], were selected for this study. In addition, the performance of the selected solvents and sorbent was evaluated at two temperature levels, 70 and 120°C. Therefore, the complete design consisted of 36 randomly performed experiments.

The study was carried out using a fortified compost sample under the following conditions: One extraction cycle of 5 minutes, at 1500 psi of pressure, flowing 6.6 mL of solvent (60% cell volume) through the extracted sample and purging with N_2 for 60 s to collect the extract. The sorbent distribution in the cell was the same in all experiments. Two cellulose filters were placed at the cell bottom and then one gram of anhydrous sodium sulphate and one gram of the corresponding sorbent. The following layer was a homogeneous

TABLE 1: MS/MS parameters for the detection of analytes and internal standard.

Compound	Time, min	Parent ion, m/z	Quantification ion, m/z	Isolation window, m/z	Ionization	ESL ^a	REV ^b
Diazinon	15.3–16.5	305	169 + 249	5	CI	120.0	86.0
Caffeine (IS)	16.5–17.5	194	193	3	EI	74.7	60.0
Iprodione	17.5–23.5	314	245 + 271	3	EI	121.0	81.0
Prochloraz	23.5–25.5	308	244 + 175	3	EI	118.8	83.0
Deltamethrin	25.5–28.0	253	172 + 174	3	EI	97.5	64.0

(a) Excitation storage level, m/z.

(b) Resonant excitation amplitude, V.

mixture of 0.5 g of sample, 1.0 g of desiccant, and 3 g of the clean-up sorbent. Finally, the cell was completely filled with anhydrous sodium sulphate and another cellulose filter was placed at the top.

Results of the experimental design are presented in Figure 2, which shows the solvent-sorbent and solvent-temperature interaction plots for the four analytes. As can be seen, high temperature provided higher recovery values in most cases but the optimal solvent-sorbent combination in terms of recovery was different for each analyte.

The best diazinon recovery values (around 70%) were obtained with acetone and alumina. Silica provided the worst results with the most solvents (recovery between 20 and 30%). Recovery values with acetone mixtures or dichloromethane as solvent and Florisil as sorbent were around 50%.

On the contrary, silica provided the best results for iprodione (50–70% recovery) with all the solvents studied but dichloromethane, and the worst combination was alumina and acetone (only 20% recovery). Florisil and alumina behaved similar but for acetone and acetone-dichloromethane; with this solvents only Florisil allowed to reach recoveries above 40%. The recovery with dichloromethane and these two sorbent was also very poor (around 25%).

Regarding prochloraz, best results were obtained with silica but only with acetone and ethyl acetate-acetone mixture as extraction solvents (recovery around 70%). Florisil and alumina provided intermediate recovery values, between 40 and 60% in most cases; and again, as in the case of iprodione, dichloromethane gave rise to the worst results with silica and Florisil.

Finally, deltamethrin recovery values were higher than 40% for all the solvent-sorbent combinations. Extraction with ethyl acetate-acetone mixture and silica as sorbent yielded a recovery higher than 70%. A similar recovery was obtained using Florisil and dichloromethane.

In one hand, the optimal combination for iprodione, prochloraz and deltamethrin was silica and the ethyl acetate-acetone mixture but it provided the worst results for diazinon (only a 20% recovery). On the other hand, the best combination for diazinon (alumina and acetone) provided poor recovery for iprodione. Moreover, Florisil combined with acetone mixtures gave rise to intermediate recovery values (45–65%) for the four pesticides. According to these results, Florisil and the 1 : 1 acetone-dichloromethane mixture were selected as a compromise situation.

Regarding to the clean-up efficiency of the sorbents studied, extracts obtained without clean-up sorbent (sorbent was replaced by sand) were highly yellow coloured, and the least coloured extracts were obtained using Florisil.

3.2.2. Influence of the Temperature, Time, and Sample-Sorbent Mass Ratio. Once the solvent and the sorbent were selected, the next step was to study the influence of other selective PLE variables such as temperature, time, and sample-sorbent mass ratio on recovery in order to select the optimal values. To accomplish this task a central composite design (CCD) was carried out. The effects of the three variables were simultaneously investigated using a 2^3 factorial design plus star points and central point. The rotatable and orthogonal central composite experimental factorial design consisted in 23 randomize experiments, corresponding to 8 cube points, 6 star points located at $\pm\alpha$ from the centre of the experimental domain, and 9 replicates of the central point. The axial distance α for this design was 1.68 in order to establish the rotatability condition.

Low and high levels for temperature, time, and mass ratio were 50 and 150°C, 1 and 15 min, and 0.04 and 0.40, respectively. Main effects, quadratic terms and two-factor interactions for variables involved in the design were calculated by analysis of variance (ANOVA). Pareto charts showing standardized values for main effects and quadratic terms are depicted in Figure 3. Dotted lines represent the upper and lower 95% confidence levels.

As can be seen in the Pareto charts, none of the variables had a significant effect in the recovery of deltamethrin and the sample-sorbent mass ratio was the only variable which affected significantly the recovery the other three target analytes (diazinon, iprodione, and prochloraz). The quadratic effect of temperature was also significant in the prochloraz recovery. Time did not affect the recovery of any of the target analytes, which means extraction was fast enough to distribution equilibrium to be reached in the lowest time value studied. Moreover, two-factor interactions were not statistically significant at the 95% confidence level in any case.

The response plots for diazinon, iprodione and prochloraz were drawn (they are included in Figure 4) considering only the variables showing significant effects. Increasing sample-sorbent mass ratio increased recovery of diazinon and iprodione and thus the highest recovery was obtained at 0.4, the highest level studied. It seems that high sorbent

FIGURE 2: Interaction plots obtained from the $6 \times 3 \times 2$ multifactor categorical experimental design.

FIGURE 3: Standardized coefficients for (a) main effects, (b) quadratic terms, and (c) crossing effect of factors considered in the CCD design. Codes: (A) mass ratio, (B) temperature ($^{\circ}\text{C}$), and (C) extraction time (min).

amounts could retain diazinon and iprodione partially. Response plot for prochloraz showed a minimum at a mass ratio value of 0.2, and a maximum at 105°C . The highest recovery value was also obtained at this temperature and a mass ratio value of 0.4.

According to these results, the conditions selected for further SPLE experiments were sample-sorbent mass ratio of 0.4 (corresponding to 1.0 g of sample and 2.5 g of Florisil), 105°C , and 2 min. Under these conditions, recovery values were around 80%.

FIGURE 4: Response plots for significant effects.

TABLE 2: Slope and regression coefficients obtained in solvent, PLE and SPLE extracts.

Compound	Ethyl acetate		PLE extract		Selective PLE extract	
	Slope $\pm$ SD	R^2	Slope $\pm$ SD	R^2	Slope $\pm$ SD	R^2
Diazinon	$(9.51 \pm 0.23)E - 03$	0.996	$(7.9 \pm 0.5)E - 03$	0.994	$(8.3 \pm 1.1)E - 03$	0.987
Iprodione	$(7.04 \pm 0.19)E - 04$	0.996	$(6.2 \pm 0.4)E - 04$	0.995	$(6.11 \pm 0.21)E - 04$	0.998
Prochloraz	$(7.85 \pm 0.21)E - 04$	0.997	$(5.34 \pm 0.06)E - 04$	0.9998	$(5.7 \pm 0.3)E - 04$	0.996
Deltamethrin	$(3.15 \pm 0.06)E - 03$	0.998	$(2.70 \pm 0.13)E - 03$	0.996	$(2.71 \pm 0.06)E - 03$	0.9992

3.2.3. In-Cell Sorbent Distribution. Until now experiments were carried out with 1.5 g of Florisil mixed with the sample and the rest (1.0 g) placed in layer behind sample. However, in this section different Florisil distributions in extraction cell were evaluated: from pressurized solid-phase extraction, (when all the sorbent are placed in a layer behind the sample) to pressurized matrix solid-phase dispersion (with all the Florisil homogeneously mixed with the sample) and also different combinations between these two situations. Different amounts of Florisil, from 0 to 2.5 g (with increments of 0.5 g) were mixed with the sample and packed in the extraction cell. The rest of Florisil until 2.5 g was added in a separated layer. Experiments were performed in triplicate. Recovery results are shown in Figure 5 and, as can be seen, highest values were obtained for 0.5 g of Florisil mixed with the sample and 2.0 g added as a separated layer. Therefore a combination of pressurized MSPD and SPE provided the best results.

3.2.4. Number of Cycles. Once the optimal conditions were established for the SPLE process, the number of static extraction steps (cycles) required for complete extraction was surveyed. The effect of one, two, or three cycles was studied. Extractions were performed in triplicate. ANOVA showed that there were not statistically significant differences among the results obtained with one, two, and three extraction cycles. F values were lower than the critical value (5.14) in all cases, ranging from 0.65 to 3.24 for diazinon and iprodione, respectively. Therefore, one extraction cycle was used to extract the four pesticides from the compost samples.

3.3. Calibration Method. In order to select the appropriate calibration method, the presence of matrix effects was investigated. For instance, coextracted compounds can affect

FIGURE 5: Influence of sorbent distribution on recovery.

ionization efficiency and thus decrease analytical signals. Calibration graph slopes (with their standard deviations) and correlation coefficients obtained for standard solutions in ethyl acetate and in compost extract are shown in Table 2. As can be seen, there are significant differences between the slope in ethyl acetate and in both PLE and SPLE compost extracts for the four pesticides; therefore standard addition method is mandatory in order to avoid matrix-effect errors.

In this analytical case, SPLE was not able to overcome the matrix effect because interference-causing compounds might be not completely retained by Florisil.

3.4. Features of the Method. First, the GC-MS/MS method was characterized for the four target pesticides in terms of linearity, limits of detection and repeatability using standard solutions in ethyl acetate. Detection limits were calculated in ethyl acetate for a signal-to-noise ratio of three ($S/N = 3$). The repeatability and intermediate precision were calculated by an ANOVA of nine replicates (three GC-MS/MS analysis replicates of a standard mixture per three days). LODs expressed in ng mL^{-1} , repeatability and intermediate precision expressed as relative standard deviation are listed in Table 3.

Then, the features of the whole analytical method (SPLE and GC-MS/MS) were obtained (Table 4). Detection limits were calculated for a signal-to-noise ratio of three in a compost extract blank, using the slope obtained in compost extract matrix and expressed as ng of analyte per g of wet compost. Repeatability and intermediate precision were calculated by ANOVA of three replicates per three days, and accuracy was assessed with a recovery study.

Recoveries were obtained at two spiking levels, 160 y 1600 ng g^{-1} , and using SPLE and PLE (without Florisil addition). Recovery values with their standard deviations ($N = 3$) are shown in Table 5. As can be seen, recovery values between 81 and 108% were obtained, and no significant differences were found between PLE and SPLE processes. However, SPLE extracts were cleaner than PLE extracts, which were strongly yellow coloured.

4. Conclusions

A method based on selective PLE and GC-MS/MS has been developed for determining four pesticides in mushroom compost samples. The selective PLE consisted in an in-cell clean-up by mixing the sample with Florisil. Best results were obtained with acetone:dichloromethane (1:1) at 105°C for 2 min and a sample-Florisil mass ratio of 0.4 g. One extraction cycle was enough to achieve complete extraction.

Although SPLE provided cleaner extracts than PLE, in-cell clean-up was not enough efficient to avoid matrix effect and therefore calibration was carried out by standard addition. The method provided good recovery values, between 81 and 103% and detection limits ranging from 4 to 6 ng g^{-1} .

Furthermore the proposed method simplified the whole analytical process as clean-up and extraction steps are integrated and that has advantages over previous methods in terms of simplicity, automation, analysis time, and solvent consumption.

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TABLE 3: Features of GC-MS/MS method.

Compound	LOD, ng mL^{-1}	Repeatability, % RSD intradays	Intermediate precision, % RSD interdays
Diazinon	0.1	11.9	10.1
Iprodione	3	6.4	8.7
Prochloraz	6	6.2	8.8
Deltamethrin	1	8.8	15.9

TABLE 4: Features of SPLE GC-MS/MS method.

Compound	Repeatability, % RSD intradays	Intermediate precision, % RSD interdays	LOD, ng g^{-1}
Diazinon	9.8	20.8	6
Iprodione	4.2	3.1	6
Prochloraz	3.2	4.1	6
Deltamethrin	4.6	4.7	4

TABLE 5: PLE and SPLE recoveries (and RSD %) at two spiking levels (160 and 1600 ng g^{-1}).

Compound	PLE		SPLE	
	160	1600	160	1600
Diazinon	105 (6)	96 (8)	93 (8)	81 (3)
Iprodione	103 (4)	93 (6)	93 (10)	92 (2)
Prochloraz	108 (6)	90 (4)	100 (6)	91 (7)
Deltamethrin	93 (4)	98 (7)	92 (6)	103 (0.6)

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