

# Efficiencies of Different Methods for Determination of Organophosphate Pesticide Residues in Fermented Wheat Substrate

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## SUMMARY

In the present work, the efficiencies of three different sample preparation methods for GC/MS determination of pirimiphos-methyl and chlorpyrifos-methyl residues in wheat (*Triticum spelta*) samples fermented by *Lactobacillus plantarum* were compared. All three methods were based on methanol:acetone=1:1 extraction, while further purification of the obtained samples was altered. First purification was through a column containing a mixture of aluminium oxide and activated charcoal slurry-packed and eluted with dichloromethane, the second was purification on a florisil column slurry-packed and eluted with ethyl acetate:acetone=4:1, while the third was based on a combination of the former two methods, i.e. clean-up through columns filled with a mixture of aluminum oxide and activated charcoal slurry-packed and eluted with ethyl acetate:acetone=4:1. The second method was found the most effective for obtaining satisfactory recoveries at four fortification levels. For pirimiphos-methyl, recoveries were in the range of 91.3-96.0% and had good reproducibility, i.e. RSD ranging from 2.2-4.1%, while the corresponding range for chlorpyrifos-methyl was 81.6-88.2%, and the RSD range 2.5-5.4%. The chosen method was further optimized in order to establish the optimum volume of elution solvent used during the clean-up procedures. The highest recoveries of 93.7±3.5% for pirimiphos-methyl and 85.3±2.5% for chlorpyrifos-methyl were obtained after elution with 25 ml volume of solvent. Considering all, simple, efficient and reliable GC/MS detection of pirimiphos-methyl and chlorpyrifos-methyl residues in wheat grain substrate altered by fermentation with *L. plantarum* was achieved by the two-steps extraction with 25 ml of methanol:acetone=1:1 solvent mix for 30 min, followed by a clean-up procedure through a glass column with florisil coupled with elution by 25 ml of ethyl acetate:acetone=4:1.

**Keywords:** Method efficiency; Pesticide determination; Pirimiphos-methyl; Chlorpyrifos-methyl; Fermented wheat

## INTRODUCTION

Pesticides have played an important role in the development of agriculture but hazards that they have brought along with them to food safety and human health have increasingly become the focus of world attention. Analyses of undesirable contaminants in amounts exceeding maximum residue limits (MRLs) in various food and feed samples is currently a problem of primary concern for quality control laboratories. There are many methods for determination of pesticide multi-residues in agricultural products and animal derived foods, but key techniques are concerned with: firstly, how residues of numerous pesticides can be thoroughly extracted from their complex matrices; secondly, how a great deal of interfering matter co-extracted with pesticides can be cleaned up; thirdly, what analytical methods should be adopted for pesticides requiring determination. As sample preparation is always a major bottleneck in all analytical procedures, many researchers have published in recent years data on pesticide analysis in a variety of food matrices.

Cereal grains are raw materials and staple commodities for food diets across the world. Cultivation and storage of grains often require an intensive use of pesticides, which may then be found in grains and in foods prepared from them (Vela et al., 2007). Cereal grains are treated with degradable pesticides, including organophosphates, carbamates, synthetic pyrethroids and insect growth regulators, both in storages and prior to shipment in order to prevent insect infestation. Currently, organophosphorus insecticides are widely used for protection of stored commodities (Collins, 2006). They are used for post-harvest treatments of wheat because of their relatively low rates of degradation under storage conditions that exist in practice. But post-harvest pesticides have been attracting much attention because their residues in stored cereal grains may be hazardous to human health (Nakamura et al., 1993; Sharma et al., 2005; Uygun et al., 2007). Among organophosphates, the most commonly used chemicals for protection of wheat/grains against insect attack during storage are: pirimiphos-methyl, an active substance with long insecticidal persistence (Fleurat-Lessard et al., 1998), and chlorpyrifos-methyl, which is listed as a product unlikely to cause acute toxicity (WHO, 1997).

Screening for traces of pesticides can be an extremely challenging task in matrices such as cereals due to large quantities of co-extracted components (e.g. starch, proteins, fats) which may adversely affect the method and instrument performance. A vitally important aspect of determination of pesticide residues in difficult matrices

is the application of an efficient extraction procedure followed by a specific final determinative step. So far, many researches have been published on method development for determination of pesticide residues in wheat grain (Bottomley and Baker, 1984; Bai et al., 2006; Pang et al., 2006; Khan et al., 2007; Walorczyk, 2008; Mastovska et al., 2010; Kolberg et al., 2011). However, as wheat flour used for bread, which is an important component of everyday diet in many countries, is subjected to biological (fermentation) and physical (baking) transformation (Sharma et al., 2005; Kaushik et al., 2009), the effectiveness of established methods for determination of pesticide residues in wheat grain becomes questionable for determination of residues in wheat matrix altered by the bread making process. Considering that the pH usually decreases below pH 5 during fermentation, while enzymes hydrolyze most of the proteins to amino acids and low molecular weight peptides, and starch is partially converted to simple sugars, which are fermented primarily to lactic acid, alcohol and carbon dioxide (Pardez-Lopez et al., 1991), it could be expected that this, as well as other significant alterations of wheat matrix, affect the efficiency of methods that have already been proved satisfactory for analysis of wheat grain.

Regarding it all, the objective of this study was to develop a rapid procedure for GC/MS analysis of pirimiphos-methyl and chlorpyrifos-methyl, the organophosphates most commonly used for protecting wheat and other grains against insect attack during storage, in fermented wheat matrix. Efficiencies of three different clean-up procedures, conducted after liquid-solid extraction using methanol:acetone=1:1, were tested. The first purification methodology was adapted from Khan et al. (2007), who developed a rapid, easy and unexpensive method for routine determination of organophosphorus residues in flour based on methanol:acetone=1:1 extraction followed by purification on columns containing a mixture of aluminum oxide and activated charcoal slurry-packed and eluted with dichloromethane. The second tested method was adopted from Đurović and Đorđević (2010), who developed a highly effective method for multiresidual determination of pesticides in soil as an extremely complex matrix, and this method, based on methanol:acetone=1:1 extraction followed by purification on florisil column and elution by ethyl acetate:acetone=4:1, has been proved effective for detection of bifenthrin residues in fermented wheat samples (Đorđević et al., 2012). The third tested method was based on a combination of the former two, i.e. after methanol:acetone=1:1 extraction, the samples were cleaned up through columns containing a mixture of aluminum oxide and activated charcoal slurry-packed and eluted with ethyl acetate:acetone=4:1.

## MATERIAL AND METHODS

### Wheat substrate

The wheat used in this study was uncontaminated grain of *Triticum spelta*, manufactured in organic production by Jevtić Farm, Bačko Gradište, Serbia. Wheat grains were milled, sterilized in autoclave at 121°C for 15 min and cooled. Slurry used as a substrate for fermentation was prepared by adding sterilized distilled water (1:1).

### Starter culture and fermentation

A probiotic strain – *Lactobacillus plantarum* (DSMZ 20174) from a collection of the Laboratory of Microbiology of the Faculty of Technology and Metallurgy, Belgrade, used in the study was maintained on MRS broth (Torlak Institute, Belgrade, Serbia) at 4–6°C. Starter culture was activated by incubation at 30°C in MRS broth using 1% inoculums until the exponential phase of growth was reached (24 h, cells diluted 1:10 gave an O.D.<sub>620nm</sub> of ca. 0.49), then harvested by centrifugation 10000 rpm for 10 min at 4°C (Velocity 14R, Dynamica, Salzburg-Mayrwies, Austria), washed with 50 mmol/l phosphate buffer pH 7.0 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), and re-suspended in sterile distilled water to its original volume, reaching cell density of about 9 log cfu/ml. The obtained fresh microbiological cultures were used for inoculation of milled wheat samples.

The wheat slurry was inoculated with 10% (v/v) starter culture (final cell number in the substrates was ca. 8 log cfu/g). Fermentation was carried out at 30°C for 48 h. Samples of fermented wheat matrix were prepared in triplicate.

### Analytical standard and working solutions

Analytical standards of pesticides were obtained from Dr. Ehrenstorfer, Augsburg, Germany (pirimiphos-methyl purity 99.2% and chlorpyrifos-methyl purity 99.9%). Stock solutions (2.0 mg/l) of standards were prepared by dissolving the weight amount in acetone. The solutions were stored at -18°C. Working standard solutions were prepared daily by diluting stocks with sterile distilled water.

Acetone, methanol and ethyl acetate, as well as anhydrous sodium sulfate (99.0% purity), aluminum oxide and activated charcoal, were purchased from J. T. Baker (Deventer, Holland), while florisil (60-100 mesh) was purchased from Serva Electrophoresis GmbH (Heidelberg,

Germany) and dichloromethane from Lachner (Praha, Czech republic). Before use, sodium sulfate was dried for 24 h at 130°C; aluminum oxide activated for 3 h at 450°C, and florisil for 4 h at 600°C and then 5 h at 130°C.

### Sample preparation and analysis

After 48 h of fermentation, sub-samples of 10 g were placed in polypropylene centrifuge tubes (Sarstedt, Germany) and one group of samples was fortified at 1, 5, 10 and 20 mg/kg level of pirimiphos-methyl and another with 0.6, 3, 6 and 12 mg/kg level of chlorpyrifos-methyl, both using 50 mg/l of working standard solutions. The spiked samples were homogenized for 3 h using a mechanical stirrer so that the pesticides were thoroughly absorbed. After adding 5 g of anhydrous sodium sulfate, these samples and a control sample (free from any pesticides) were then extracted twice with 25 ml of a methanol:acetone=1:1 solvent mix for 30 min on a rotary stirrer and then centrifuged for 3 min at 4000 rpm (UZ 4, Iskra, Slovenia). The extracts were filtered through a filter paper containing 1 g of anhydrous sodium sulfate and evaporated to dryness at 35°C using a rotary evaporator (Devarot, Elektromedicina, Slovenia).

The obtained residues were further processed through three different purification procedures and finally analyzed by GC/MS.

In the first purification procedure (M1), the residues were re-dissolved in 2.5 ml of dichloromethane. Of the obtained solutions, 2 ml were passed through a glass column containing 1 g of sodium sulfate and 4 g of a mixture of aluminum oxide and activated charcoal (12:1) slurry-packed with 25 ml of dichloromethane. The pesticides were eluted by 25 ml of dichloromethane. Eluates were evaporated to dryness and re-dissolved in 2 ml of acetone for GC-MS analysis.

In the second clean-up procedure (M2), the residues were re-dissolved in 2.5 ml of ethyl acetate:acetone=4:1 mixture. Of the obtained solutions, 2 ml were passed through a glass column containing 1 g of sodium sulfate and 5 g of florisil slurry-packed with 25 ml of ethyl acetate:acetone=4:1 mixture. The pesticides were eluted by 25 ml of ethyl acetate:acetone=4:1. Eluates were evaporated to dryness and re-dissolved in 2 ml of acetone for GC-MS analysis.

In the third purification procedure (M3), the residues were re-dissolved in 2.5 ml of ethyl acetate:acetone=4:1 mixture. Of the obtained solutions, 2 ml were passed through a glass column containing 1 g of sodium sulfate and 4 g of mixture of aluminum oxide and activated charcoal (12:1) slurry-packed with 25 ml of ethyl

acetate:acetone=4:1 mixture. The pesticides were eluted by 25 ml of ethyl acetate:acetone=4:1. Eluates were evaporated to dryness, and re-dissolved in 2 ml of acetone for GC-MS analysis.

In order to rationalize the utilization of solvents, the chosen method was further optimized by changing the elution solvent volume used during the clean-up procedures. Efficiencies of the clean-up procedure through the glass column with florisil, followed by elution by 15, 20, 25, 30 and 35 ml of ethyl acetate:acetone=4:1 (after two steps of extraction with 25 ml of methanol:acetone=1:1 solvent mix for 30 min) were tested.

Fermented wheat grain free of pesticides and extracted with the same procedures was used for preparation of calibration standards for quantitative determination of pirimiphos-methyl and chlorpyrifos-methyl. The efficiencies of three described sample preparation methods were evaluated by determining the recoveries of pesticides at four different fortification levels. Additionally, in the most effective method, the optimum elution solvent volume (between 15 and 35 ml) within sample clean-up procedure was optimized too.

### Instrumentation

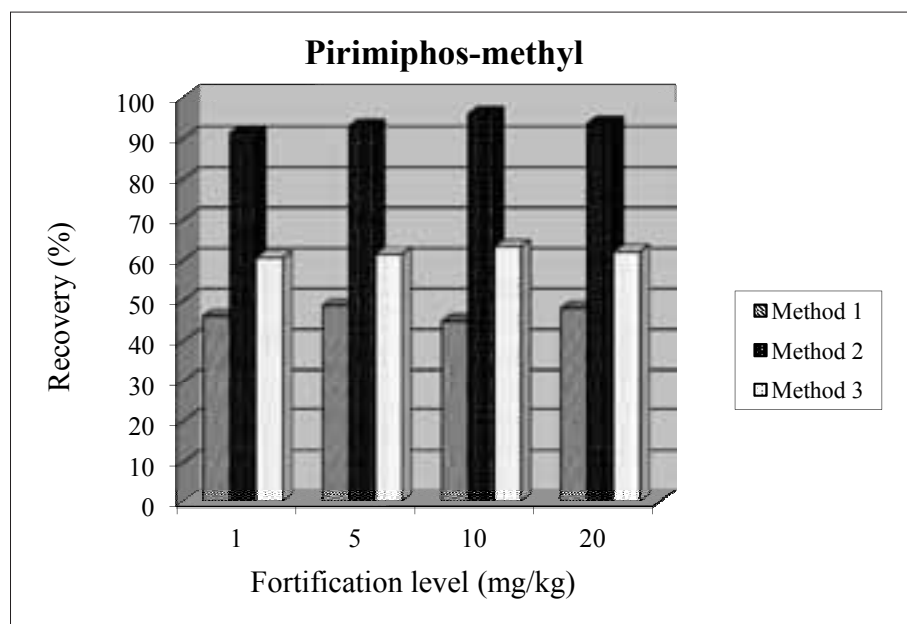
A gas chromatograph-mass spectrometer (GC/MS) was used as a detection device (CP-3800/Saturn 2200, Varian Australia) with 30 m x 0.25 mm x 0.25  $\mu$ m, VF-5ms

column (Varian, Australia). The GC was programmed as follows: initial temperature was 170°C, then increased to 260°C at 9°C/min and held for 3.5 min. The carrier gas (helium, 99.999%) flow rate was in constant flow mode at 1.1 ml/min. The ion trap mass spectrometer was operated in the electron impact/selected ion monitoring (EI/SIM) mode. Ions ( $m/z$ ) used for quantification (confirmation) were: 290(233) for pirimiphos-methyl and 286(125) for chlorpyrifos-methyl. The ion trap and transferline temperatures were set to 210°C and 250°C, respectively.

### RESULTS AND DISCUSSION

For each method used, recovery tests were performed at four fortification levels: 1, 5, 10 and 20 mg/kg for pirimiphos-methyl and 0.6, 3, 6 and 12 mg/kg for chlorpyrifos-methyl. Concentrations of 5 mg/kg and 3 mg/kg were chosen to meet respectively pirimiphos-methyl and chlorpyrifos-methyl maximum residue levels (MRLs) for wheat, as established by the national and international regulations (Pravilnik, Službeni Glasnik RS 25/10; Council Directive 91/414/EEC).

The results presented in Figure 1 and Figure 2 show that the recoveries differed substantially among the methods at all concentration levels for both pesticides, while differences between individual methods due to pesticide concentration at all spiking levels were minimal.



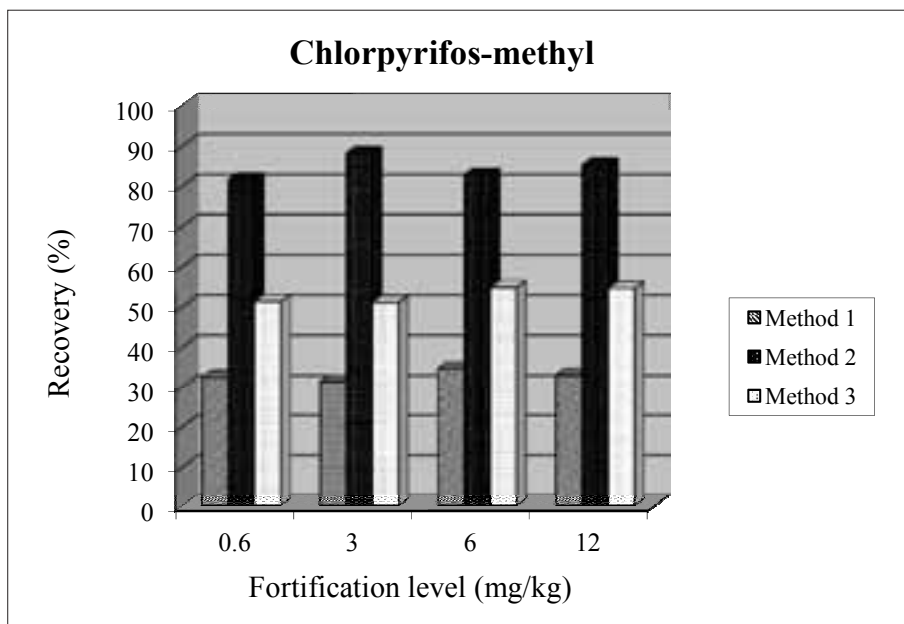
**Figure 1.** Efficiency of sample preparation methods used for determination of pirimiphos-methyl in fermented wheat samples

The average recoveries for pirimiphos-methyl determined by the first method (M1) were within a low range of 44.9–48.8%, i.e. 46.2, 48.8, 44.9 and 48.2% for the four respective fortification levels (Figure 1), with relatively good reproducibility, i.e. RSD of 3.9, 3.5, 4.0 and 2.5%. For chlorpyrifos-methyl, those recoveries were even lower, in the range of 31.3–34.6%, i.e. 32.4, 31.3, 34.6 and 32.9% for the four fortification levels respectively (Figure 2) with somewhat lower but good reproducibility, i.e. RSD 8.7, 9.2, 5.0 and 3.8%. These results demonstrate that, although Khan et al. (2007) had shown that the method was effective and reliable for determination of organophosphorus pesticide in wheat flour, it was found not to recover the used organophosphates properly from the fermented wheat samples. Therefore, the procedure, developed as a simple, rapid and economic procedure for multi-residue analysis of pesticides in wheat flour and used for routine monitoring, cannot be used for determination of pirimiphos-methyl and chlorpyrifos-methyl residues in wheat substrate altered by fermentation. Apparently, low pH value, coupled with other changes in wheat substrate, affects the efficiency of this method even though it has been proved satisfactory for analysis of wheat grain.

On the other hand, recoveries for pirimiphos-methyl obtained after the second sample preparation method (M2) were in the range of 91.3–96%, precisely 91.3, 93.0, 96.0 and 93.7% respectively for the four fortification

levels (Figure 1) and with good reproducibility, i.e. RSD of 4.1, 2.7, 2.2 and 3.5%. Also, chlorpyrifos-methyl recoveries, although some of them lower, were still in a very high range of 81.6–88.2% for all fortification levels, i.e. 81.6, 88.2, 82.8 and 85.3% (Figure 2) with good reproducibility, i.e. RSD of 5.4, 4.6, 2.7 and 2.5%, respectively. This method, proposed by Đurović and Đorđević (2010) as one that can be used for efficient determination of various pesticides in soil as one of the most complex matrices and successfully applied for bifenthrin residue analysis in fermented wheat samples (Đorđević et al., 2012), allows also a simple, efficient and reliable detection of pirimiphos-methyl and chlorpyrifos-methyl residues in wheat grain fermented by *Lactobacillus plantarum*. It seems that the proposed clean-up approach on florisil permits simultaneous removal of a broad range of compounds from fermented wheat matrices, which results in reduced matrix enhancement effect and interferences, and increase in sensitivity, permitting residues determination at the required levels.

The third tested method (M3) was developed based on a combination of the former two. The mixture of acidic alumina and charcoal was maintained as a clean-up column filling, considering that acidic alumina is one of the best matrices in its capacity for retaining oils, while charcoal effectively retains coloring coextractives, and the considerably toxic dichlormethane was replaced with a mixture of less toxic ethyl acetate and acetone for



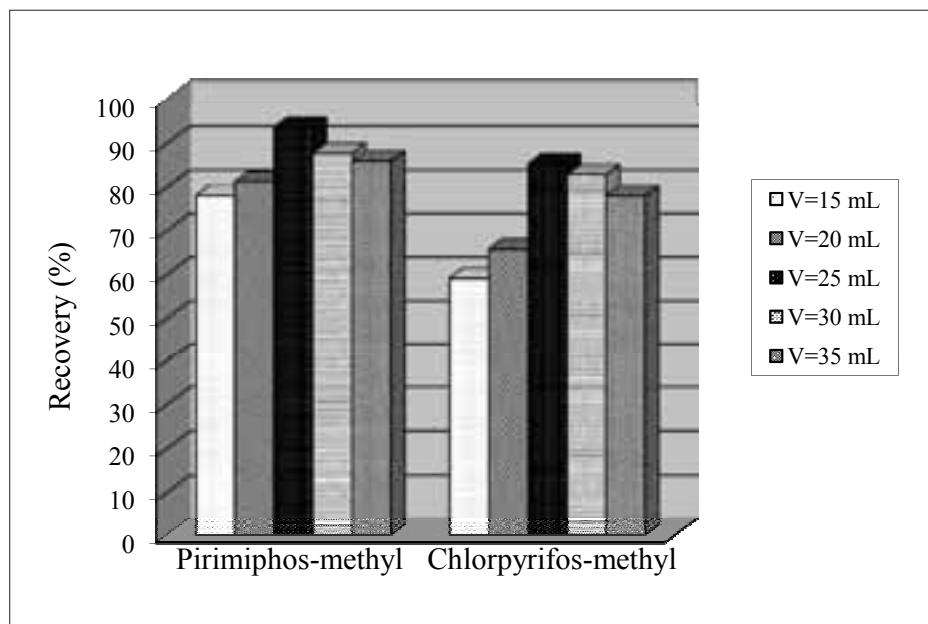
**Figure 2.** Efficiency of sample preparation methods used for determination of chlorpyrifos-methyl in fermented wheat samples

elution through columns. After such sample preparation, the obtained recoveries for pirimiphos-methyl and chlorpyrifos-methyl were significantly higher than recoveries after M1, but notably lower than those resulting from M2. For pirimiphos-methyl, the recoveries were in the range 60.5–63.3%, i.e. 60.5, 61.4, 63.3 and 62.0%, respectively, for the four fortification levels (Figure 1) with good reproducibility, i.e. RSD of 4.0, 2.7, 3.3 and 2.6%. Recoveries for chlorpyrifos-methyl were somewhat lower (51.1–54.8%), compared with pirimiphos-methyl recoveries, which is in correlation with lower recoveries obtained after the first and second tested sample preparation methods. For the four fortification levels, those recoveries were 51.3, 51.1, 54.8 and 54.6% respectively with reproducibility of 4.9, 2.7, 5.8 and 3.5%. It is possible that retention of the tested pesticides was stronger on the sorbent used in this method, so that the eluting solvent mix was not suitable enough for those analytes.

Comparing the results obtained from the three tested sample preparation methods we concluded that the second method, i.e. the combination of florisil and ethyl acetate:acetone=4:1, was the most effective for obtaining satisfactory recoveries in the analysis of tested organophosphate residues from wheat fermented with lactobacilli. The limits of detection (LOD) and quantitation (LOQ) of this method were determined according to IUPAC recommendations (Currie, 1999) as  $3.29 \times s_B$  and  $16.67 \times s_B$ , where  $s_B$  is the blank standard deviation. The obtained

LOD and LOQ were respectively 0.011 mg/kg and 0.04 mg/kg for pirimiphos-methyl, and 0.007 mg/kg and 0.04 mg/kg for chlorpyrifos-methyl. Considering that MRLs for those pesticides in wheat are 5 and 3 mg/kg (Pravilnik, Službeni Glasnik RS 25/10; Council Directive 91/414/EEC), it is obvious that the presented method is sensitive enough for determination of these organophosphates at concentration levels much below their MRL values.

In order to rationalize the utilization of solvents, the chosen method was further optimized by changing the elution solvent volume used during clean-up procedures. Efficiencies of elution with 15, 20, 30 and 35 ml solvent volumes were tested after the 25 ml ethyl acetate:acetone=4:1 elution mix. This additional study was performed at the highest fortification level (20 mg/kg for pirimiphos-methyl and 12 mg/kg for chlorpyrifos-methyl), and samples were prepared in triplicates. As presented in Figure 3, the obtained recoveries for pirimiphos-methyl were in the range of 78.1–93.7% with significant differences. Thus, the lowest recovery of 78.1% (with RSD of 2.7%) was obtained after elution with the smallest volume of solvent (15 ml), followed by somewhat but not significantly higher recovery of 81.3% (with RSD of 3.3%) obtained after elution with 20 ml of solvent. After elution with 30 and 35 ml of solvent, the recoveries were 87.8 and 86.0%, respectively (with RSDs of 1.8 and 2.5%). These results do not differ significantly among each other but are significantly higher from those



**Figure 3.** Efficiency of sample preparation methods depending on the elution solvent volume used during clean-up procedure

obtained after elution with 15 and 20 ml of solvent, and significantly lower from the recovery after elution with 25 ml ( $93.7 \pm 3.5\%$ ). Based on the results, the volume of 25 ml of solvent mix was chosen as optimal for sample preparation procedures in further investigation. As for chlorpyrifos-methyl, the lowest recovery of 59.2% (with RSD of 2.9%) was obtained after elution with the smallest volume of solvent (15 ml), followed by significantly higher recovery of 65.9% (with RSD of 3.7%) obtained after elution with 20 ml of solvent. After elution with 35 ml of solvent the recovery was  $78.1 \pm 2.6\%$ , which was significantly lower than the one obtained after elution with 30 ml of solvent ( $82.9 \pm 2.5\%$ ). Recovery obtained after elution with 25 ml was 85.3% (with RSD of 2.5%), which was not significantly different from the one obtained after elution with 30 ml of solvent, but, since it was still somewhat higher and due to practical and environmental reasons, the volume of 25 ml of solvent mix was chosen as optimal for the sample preparation procedure in further investigation.

To summarize, two repeated extractions with 25 ml of methanol:acetone=1:1 solvent mix for 30 min, followed by a clean-up procedure through a glass column with florisil coupled with elution by 25 ml of ethyl acetate:acetone=4:1, enable simple, efficient and reliable detection of pirimiphos-methyl and chlorpyrifos-methyl residues in wheat grain fermented by *Lactobacillus plantarum*.

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## Efikasnost različitih metoda za određivanje ostataka organofosfata u fermentisanim uzorcima žita

### REZIME

Poređene su efikasnosti tri različite metode pripreme uzoraka za određivanje ostataka pirimifos-metila i hlorspirifos-metila u uzorcima pšenice (*Triticum spelta*) fermentisane sa *Lactobacillus plantarum*. Sve tri metode zasnivale su se na metanolsko-acetonskoj (1:1) ekstrakciji dok su se procedure prečišćavanja dobijenih ekstrakata razlikovale. Prva metoda bila je prečišćavanje kroz kolonu punjenu smešom aluminijum-oksida i aktivnog uglja u kombinaciji sa dihlormetanom kao eluentom, druga prečišćavanje kroz kolonu punjenu florisilom u kombinaciji sa smešom etil-acetata i acetona (4:1) kao eluentom, dok se treća zasnivala na kombinaciji prve dve, tj. prečišćavanju kroz kolonu punjenu smešom aluminijum-oksida i aktivnog uglja u kombinaciji sa smešom etil-acetata i acetona (4:1) kao eluentom.

Druga testirana metoda pokazala se kao najefikasnija, pri čemu su dobijeni prinosi pirimifos-metila u opsegu 91,3-96,0% za četiri koncentraciona nivoa obogaćivanja, sa RSD% u opsegu 2,2-4,1%, dok su za hlorspirifos-metil ovi prinosi bili u opsegu 81,6-88,2% sa RSD% u opsegu 2,5-5,4%. Odabrana metoda je dodatno optimizovana variranjem različitih zapremina korišćenog eluenta. Najveći prinos metode za oba ispitivana pesticida postignut je pri zapremini eluenta od 25 ml ( $93,7 \pm 3,5\%$  za pirimifos-metil i  $85,3 \pm 2,5\%$  za hlorspirifos-metil). Predložena metoda, bazirana na 30-minutnoj ekstrakciji sa 25 ml smeše metanol:acetona=1:1, prečišćavanju na florisilskoj koloni i eluiranju sa 25 ml smeše etil-acetat:acetona=4:1, pokazala se kao efikasna, jednostavna i pouzdana metoda za određivanje ostataka ispitivanih organofosfata u fermentisanom žitnom supstratu.

**Ključne reči:** Efikasnost metode; određivanje pesticida; pirimifos-metil; hlorspirifos-metil; fermentisana pšenica