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Comparison of Methods for Bifenthrin Residues Determination in Fermented Wheat Samples

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ABSTRACT

Efficiency of three different sample preparation methods for GC/MS determination of bifenthrin residues in wheat (*Triticum spelta*) samples fermented by *Lactobacillus plantarum* was tested. The first method was based on a methanol:acetone=1:1 extraction folowed by a purification on columns containing mixture of aluminium oxide and activated charcoal slurry-packed and eluted with dichlormethane, the second was based on methanol:acetone=1:1 extraction folowed by the purification on florisil column and elution by ethil acetate:acetone=4:1, while the third tested method was based on a combination of the first two mentioned methods, thus methanol:acetone=1:1 extraction and clean-up throught columns filled with a mixture of aluminum oxide and activated charcoal slurry-packed and eluted with ethil acetate:acetone=4:1. The second method was the most effective for obtaining satisfactory recoveries for bifenthrin in a range of 79-83% for four fortification levels, with good reproducibility i.e. RSD% in a range of 2.2-7.4%. The chosen method was further optimized by assessing the optimum volume of elution solvent used during the clean-up procedures. The highest recovery of 82.1% was obtained after elution with 25 ml of solvent. Overall, two-step extraction with 25 ml of methanol:acetone=1:1 solvent mix for 30 min, followed by clean-up procedure through a glass column with florisil coupled with elution with 25 ml of ethyl acetate: acetone=4:1, allows simple, efficient and reliable GC/MS detection of bifenthrin residues from wheat grain fermented by *L. plantarum*.

Keywords: Wheat; Fermentation; Pesticides; Residues

INTRODUCTION

As wheat is a major cereal grain cultivated throughout the world with one third of yield consumed by milling and baking (Kihlberg et al., 2004), this cereal in the form of flour and its processed products has become an essential part of human diet. Continuous increase of wheat productivity can be attributed not only to the increase in cultivated area, but also to a yield increase which is related to the intensive use of agrochemicals. Therefore, the presence of toxic pesticide residues in wheat and related food products is a real and important concern to human health.

Generally, wheat grain becomes contaminated with pesticides from two principal sources, pesticide residues originating from field spraying and the accumulated residues from pesticide treatments during storage (Jamil et al., 2005; Iqbal and Ali, 2006). A large proportion of stored cereal grain is protected against insect attack by residual pyrethroid insecticides, as a cost effective successful alternatives to traditional organophosphorous insecticides. Their popularity for insect control is based on several properties, including limited environmental persistence, high insecticidal activity and low mammalian toxicity (Martinez-Galera et al., 2003; Khambay and Jewess, 2005). Moreover, residual pyrethroid insecticides as grain protectants can be applied easily without specialized equipment. They are compatible with international cereal trade and global restrictions for zero insect tolerance, and are effective against a wide range of storage pests (Arthur, 1996). Therefore, it is likely that pyrethroid grain protectants will be a valuable tool in pest management programs in the future as well (White and Leesch, 1995; Arthur, 1996).

The control of pesticide residues in grain is generally based on MRLs, which are below the highest expected residue levels if the registered dosages are applied according to good agricultural practice. However, although MRLs are a credible and useful means for regulating the acceptable pesticide use, they are not sufficient for the assessment of human health risks from residue intake unless accurate knowledge about the loss of residues in the processing treatment is available (Fleurat-Lessard et al., 2007). A lot of publications related to the fate of pyrethroids on a raw grain material have been published (Morillo et al., 2001; Khan et al., 2007; Sonchieu et al., 2010; Uddin et al., 2011). However, little work has been conducted on the fate of these compounds during the grain processing. In the context of a recent increased interest in pesticide residue levels in processed food, it is interesting to study the loss of pesticides during the fermentation as one of the oldest biotechnological processes.

Fermentation is a simple process during which the enzymes hydrolyze most of the proteins into amino acids and low molecular weight peptides, and partially convert starch to simple sugars which are fermented primarily to lactic acid, alcohol and carbon dioxide lowering the pH typically below pH 5 (Pardez-Lopez et al., 1991). Reduction in pesticide residues during fermentation has been continuously studied in different food commodities (Abou-Arab, 2002; Sharma et al., 2005; Navarro et al., 2006, 2007; Rajashekar et al., 2007; Garau, 2009; Jung et al., 2009; Bo and Zhao, 2010; Čuš, 2010), but there is a lack of data regarding determination of pesticide residues in sourdough fermented wheat, i.e. its dissipation during fermentation process.

To the present, many researches have been published on the development of methods for determination of pesticide residues in wheat grain (Bottomley and Baker, 1984; Nakamura et al., 1993; Pang et al., 1995a, 1995b, 1997, 1999; Kaushik and Handa, 1997; Guo-Fang et al., 2006; Khan et al., 2007). However, little work has been conducted concerning efficiency of those methods in determination of pyrethroid residues in wheat matrix altered by fermentation. As mentioned before, during sourdough fermentation pH decreases below pH 5, and it could be expected that this, as well as other significant alterations of a matrix, affect the efficiency of methods that have already proved to be satisfactory for analysis of wheat grain.

Considering the abovementioned, objective of this study was to develop a rapid procedure for GC/MS analysis of bifenthrin, as one of the most commonly used active substance in plant protection in our country (Janjić and Elezović, 2010), in fermented wheat matrix. The efficiency of three different clean up procedures, after liquid–solid extraction using methanol: acetone=1:1, were tested. The first purification methodology was adapted from Khan et al. (2007) who developed an easy and unexpensive method for determination of pyrethroid residues in flour, based on methanol:acetone=1:1 extraction folowed by a purification on columns containing mixture of aluminum oxide and activated charcoal slurry-packed and eluted with dichlormethane. The second tested metod was adopted from Đurović and Đorđević (2010) who developed a highly effective method for multiresidual determination of pesticides in soil as an extremely complexed matrix, based on methanol:acetone=1:1 extraction folowed by the purification on florisil column and elution

by ethil acetate:acetone=4:1. The third tested method was based on a combination of first two mentioned methods, thus after methanol:acetone=1:1 extraction, samples were clened-up throught columns containing mixture of aluminum oxide and activated charcoal slurry-packed and eluted with ethil acetate:acetone=4:1.

MATERIAL AND METHODS

Starter culture

A probiotic strain – *Lactobacillus plantarum B28* used in the study was obtained from collection of the Laboratory of Microbiology of the Faculty of Technology and Metallurgy, Belgrade. The strain was maintained on MRS broth (Torlak Institute of Immunology and Virology, Belgrade, Serbia) at 4-6°C. Starter culture was activated by 24 h incubation at 30°C in MRS broth using 1% inoculums. The culture was centrifuged (10000 rpm, 10 min, 4°C), washed in phosphate buffer solution pH 7.0 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and re-suspended in distilled water to its original volume. The obtained fresh microbiological cultures were used for inoculation of milled wheat samples.

Wheat substrate

Wheat mash used as a substrate in this study was prepared from uncontaminated grain of *Triticum spelta* manufactured in organic production by Jevtić farm, Bačko Gradište, Serbia. Wheat grains were milled, sterilized in autoclave for 20 minutes and cooled, and slurry was prepared by adding distilled water (1:1).

Fermentation

The wheat mash was inoculated with 10% (v/v) starter culture. 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 standard of bifenthrin was obtained from Dr. Ehrenstorfer, Augsburg, Germany (purity 98.4%). Stock solution (1.0 mg/l) of standard was prepared by dissolving the weight amount in acetone. The solution was stored at -18°C. Working standard solutions were prepared daily by diluting stock 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 (Germany) and dichloromethane from Lachner (Czech Republic). Before use, sodium sulfate was dried 24 h at 130°C; aluminum oxide activated 3 h at 450°C, and florisil 4 h at 600°C and then 5 h at 130°C.

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 μm, 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 operated in the electron impact/selected ion monitoring (EI/SIM) mode. Ion (m/z) 181 was used for quantification, while ion (m/z) 166 was used for confirmation. The ion trap and transferline temperatures were set to 210°C and 250°C, respectively.

Sample preparations and analysis

After 48 h of fermentation, 10 g of sub-samples were placed in polypropylene centrifuge tubes (Sarstedt, Germany) and fortified at 0.5, 1.0 and 2.0 mg/kg rates of bifenthrin using 50 mg/l working standard solution. The spiked samples were homogenized for 3h using a mechanical stirrer so the pesticide was thoroughly absorbed. These samples along with a control sample (the sample free from any pesticides) were then, afer adding the 5 g of anhydrous sodium sulfate, extracted two times with 25 ml of 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 residues were further processed through three following procedures and finally analyzed by GC/MS.

In the first purification procedure (M1) the residues were re-dissolved in 2.5 ml of dichlormethane and 2 ml of obtained solutions 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).

The sorbent was transferred to column using 25 ml of dichlormethane. The pesticides were eluted with 25 ml of dichloromethane. The eluates were evaporated to dryness, and re-dissolved in 2 ml of acetone for GC-MS analysisis.

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

In the third purification procedure (M3) the residues were re-dissolved in 2.5 ml of ethyl acetate:acetone=4:1 mixture, and 2 ml of obtained solutions 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). The sorbent was transferred to column using 25 ml of ethyl acetate: acetone=4:1 mixture. The pesticides were eluted with 25 ml of ethyl acetate: acetone=4:1. The eluates were evaporated to dryness, and re-dissolved in 2 ml of acetone for GC-MS analysisis.

Fermented wheat grains free of bifenthrin and extracted with the same procedures were used for preparation of calibration standards for quantitative determination of bifenthrin. The efficiency of three described sample preparation methods was evaluated by determining the recoveries of bifenthrin 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 also optimized.

RESULTS AND DISCUSSION

For every method used, recovery tests were performed at four fortification levels: 0.1, 0.5, 1 and 2 mg/kg. The rate of 0.5 mg/kg was chosen to meet bifenthrin maximum residue level (MRL) for wheat, established by the national and international regulations (Službeni glasnik RS 25/10; Council Directive 91/414/EEC).

As it can be seen from the results presented on Figure 1, the obtained recoveries differed substantially among methods at all concentration levels, while differences within individual methods regarding bifenthrin concentration at all spiking levels were minimal.

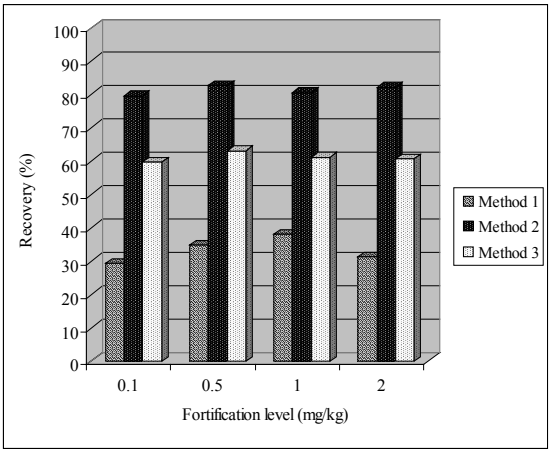

Figure 1. Efficiency of sample preparation methods used for determination of bifenthrin in fermented wheat samples

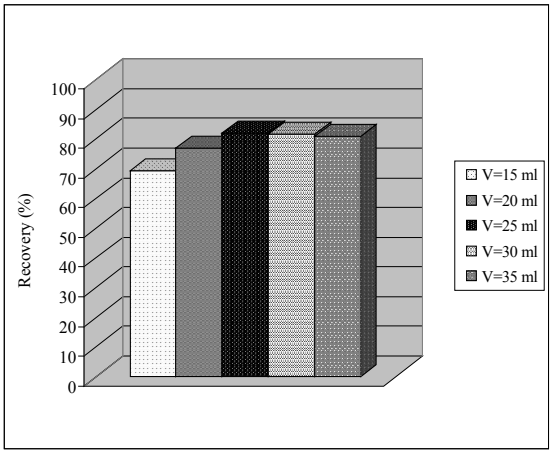

Figure 2. Dependence of sample preparation efficiency on volumes of elution solvent used during the clean-up procedure. Two-step extraction with 25 ml of methanol:acetone=1:1 solvent mix for 30 min, followed by the second clean-up procedure through a glass column with florisil coupled with elution with ethyl acetate:acetone=4:1 was used.

The average recoveries for bifenthrin determined by the first method (M1) were in a very low range 29 – 38% i.e. 29.3, 34.9, 38.0 and 31.3% respectively, for four fortification levels (Figure 1), with relatively good reproducibility i.e. RSD% of 10.3, 5.6, 9.7 and 4.5%. These results demonstrate that this method does not recover bifenthrin properly from fermented wheat samples with low pH value, although Khan et al. (2007) indicated that method was effective and reliable for determination of this pesticide in wheat flour. Thus, though

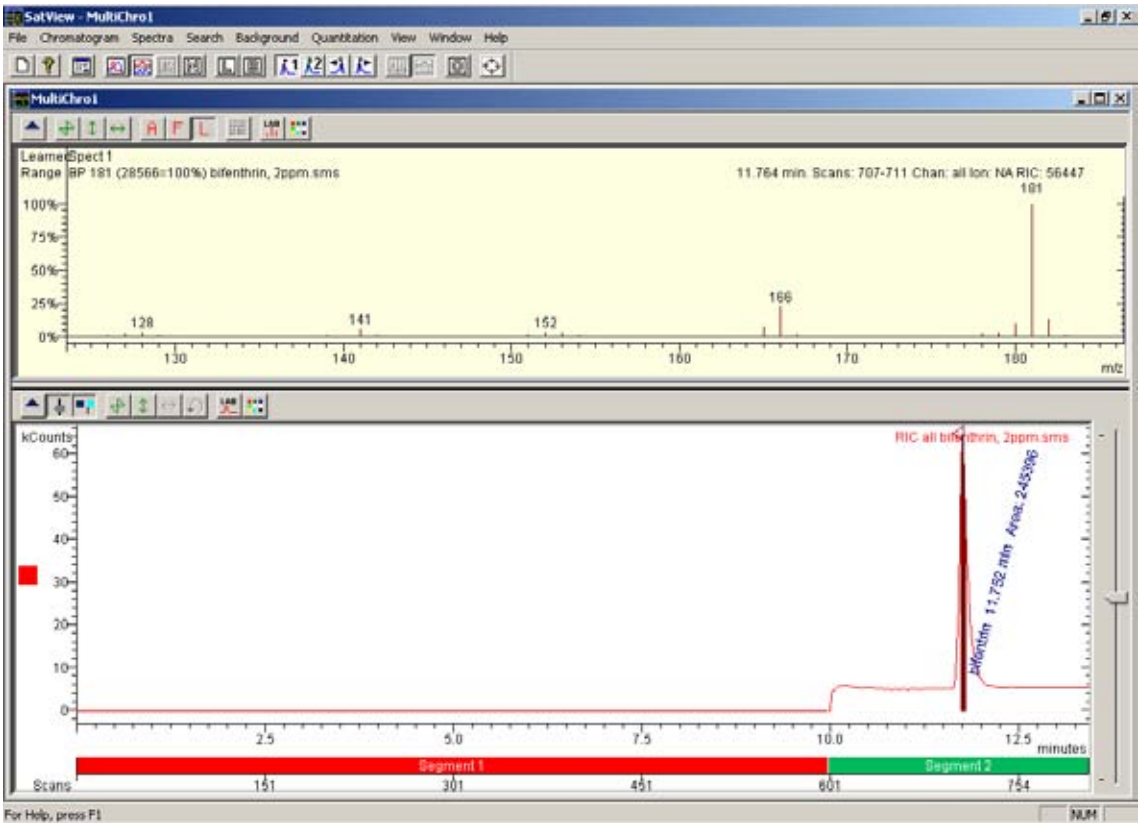

Figure 3. Both, GC/MS chromatogram and spectra of fermented wheat extract spiked at 2 mg/kg bifenthrin rate. Extract was obtained by two step extraction with 25 ml of methanol:acetone=1:1 solvent mix for 30 min, followed by clean-up procedure through a glass column with florisil coupled with elution with 25 ml of ethyl acetate:acetone=4:1

mentioned group of authors managed to develop a simple, rapid and economic procedure for analysis of multi-residue pesticides in wheat flour for routine monitoring, this procedure could not be used for the determination of bifenthrin residues in wheat substrate altered by fermentation.

On the contrary, the recoveries for bifenthrin obtained after the second sample preparation method (M2) were in the range of 79-83%, precisely 79.6, 82.6, 80.5 and 82.1% respectively for four fortification levels (Figure 1), with good reproducibility i.e. RSD% of 7.4, 4.1, 2.2 and 3.8%. It can be seen that this method, proposed by Đurović and Đorđević (2010) as one that can be used for efficient determination of various pesticides from one of the most complexed matrix samples such as soil, could also be applied to bifenthrin residues analysis in fermented wheat samples.

The third tested method (M3) was developed based on a combination of the first two mentioned methods. As acidic alumina is one of the best matrixes regarding its capacity for retaining oils, while charcoal efectively retains coloring coextractives, mixture of those was maintained as clean-up column filling. On the other side, dichloromethane, as considerably toxic solvent, was replaced with a mixture of less toxic ethyl acetate and acetone for elution through columns. The recoveries for bifenthrin obtained after this sample preparation method were significantly higher than recoveries from M1, but notably lover than those from M2, and were in the range of 59-63% i.e. 59.7, 63.0, 61.1 and 60.6% respectively for four fortification levels (Figure 1), with good reproducibility i.e. RSD% of 5.9, 4.2, 6.4 and 10.4%.

As a conclusion, the second method, with the combination of florisil and ethyl acetate:acetone=4:1, was the most effective for obtaining satisfactory recoveries for bifenthrin residues analysis from wheat fermented with lactobacilli. The limits of detection (LOD) and quantification (LOQ) of this method were determined according to IUPAC recommendations (Currie, 1999),

as 3.29 x s_B and 16.67 x $s_{B,,}$ where s_B is the blank standard deviation. The obtained LOD and LOQ were 0.004 mg/kg and 0.014 mg/kg, respectively. Considering that MRL for bifenthrin in wheat is 0.5 mg/kg (Službeni glasnik RS 25/10; Council Directive 91/414/EEC), it is obvious that the presented method is sensitive enough for determination of this pesticide at concentration levels much below its MRL value.

The chosen method was further optimized by assessing the optimum volume of elution solvent used during the clean-up procedures. Besides previously used 25 ml of ethyl acetate: acetone=4:1 elution mix the efficiency of elution with 15, 20, 30 and 35 ml of solvent were also tested. This additional study was performed at the highest fortification level (2 mg/kg), and samples were prepared in triplicates. As presented in Figure 2, the obtained recoveries were in the range of 69-82% with significant differences. Thus, the lowest recovery of 69.30% (with RSD% of 3.8%) was obtained after the elution with the smallest volume of solvent (15 ml), followed by the recovery of 76.9% (with RSD% of 4.1%) obtained after the elution with 20 ml of solvent. After the elution with 30 and 35 ml of solvent the recoveries were 81.6 and 81.0% respectively (with RSD% of 4.6 i.e. 3.3%). These results did not differ significantly from those obtained after the elution with 25 ml of solvent (82.1%), but, since they are still somewhat lower, and due to practical and environmental reasons, volume of 25 ml of solvent mix was chosen as optimal within sample preparation procedure for further investigations.

Overall, two repeated extractions with 25 ml of methanol:acetone=1:1 solvent mix for 30 min, followed by clean-up procedure through a glass column with florisil coupled with the elution with 25 ml of ethyl acetate: acetone=4:1, allow simple, efficient and reliable detection of bifenthrin residues from wheat grain fermented by *Lactobacillus plantarum*, providing representative GC-MS chromatograms (Figure 3).

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Poređenje metoda za određivanje ostataka bifentrina u fermentisanim uzorcima žita

REZIME

Testirane su efikasnosti tri različite metode pripreme uzoraka za određivanje ostataka bifentrina u uzorcima pšenice (*Triticum spelta*) fermentisane pomoću *Lactobacillus plantarum*. Prva metoda zasnivala se na metanolsko-acetonskoj (1:1) ekstrakciji i prečišćavanju kroz kolonu punjenu smešom aluminijum-oksida i aktivnog uglja u kombinaciji sa dihlormetanom kao eluentom, druga se zasnivala na metanolsko-acetonskoj (1:1) ekstrakciji i prečišćavanju 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. metanolsko-acetonskoj (1:1) ekstrakciji i prečišćavanju kroz kolonu punjenu smešom aluminijum-oksida i aktivnog uglja u kombinaciji sa smešom etil-acetata i acetona (4:1) kao eluentom. Pokazalo se da je druga testirana metoda najefikasnija, pri čemu su dobijeni prinosi bifentrina u opsegu 79-83% za četiri koncentraciona nivoa obogaćivanja, sa RSD% u opsegu 2,2-7,4%. Odabrana metoda je dodatno optimizovana variranjem različitih zapremina korišćenog eluenta. Najveći prinos metode postignut je pri zapremini eluenta od 25 ml. Predložena metoda, bazirana na 30-minutnoj ekstrakciji sa 25 ml smeše metanol:aceton=1:1, prečišćavanju na florisilskoj koloni i eluiranju sa 25 ml smeše etil-acetat:aceton=4:1, pokazala se kao efikasna, jednostavna i pouzdana metoda za određivanje ostataka bifentrina u fermentisanom žitnom supstratu.

Ključne reči: Pšenica; fermentacija; pesticidi; ostaci