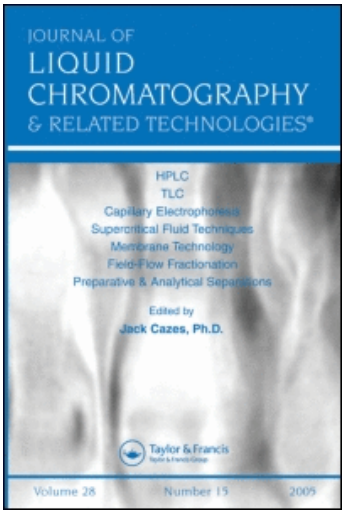

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Comparison of Various Extraction Techniques to Determine Fungicide Residue in Wheat Grain

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Comparison of Various Extraction Techniques to Determine Fungicide Residue in Wheat Grain

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Abstract: Methods for the extraction, separation, detection, and quantification of benzimidazole fungicide carbendazim residue in wheat grain were evaluated. The extraction of the residue was achieved using liquid–liquid extraction (LLE), solid-phase extraction (SPE), and matrix solid phase dispersion (MSPD). Their respective advantages and disadvantages were discussed. Determination was carried out by reversed-phase high performance liquid chromatography (RP-HPLC) with column switching and diode array detection (DAD). Recoveries, at spiked concentrations below the maximum acceptable residue levels established by the Polish Government, were between 71.2–76.5% for LLE, 82.2–83.2% for SPE, and 84.3–90.7% for MSPD. Relative standard deviations (RSDs) ranging from 5.2% for LLE, 3.1–4.6% for SPE, and 2.7–4.1% for MSPD. The limit of quantification (LOQ) at $\lambda = 279$ nm was 0.02 μg/g for all the extractions. Results obtained by the methods were compared in terms of sensitivity and selectivity and the three methods were applied to analyze real samples. As MSPD is easier to perform, faster than the organic solvent extraction,

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and shows equal accuracy and resolution, its application for analyzing pesticides in wheat is recommended.

Keywords: Sample preparation, Pesticide residue, Fungicide, Carbendazim, Plant matrix, HPLC determination

INTRODUCTION

Pesticides are widely utilized at various stages of cultivation and during post-harvest storage to protect fruit and vegetables against a range of pests and fungi and/or to provide quality preservation. The risk of pesticide residues depends on their ability to cause adverse health effects and the potential human exposure to their residues in the diet.[1] There is a strict legislative framework controlling the use of such substances with the aim of minimizing the risk to human health associated with the consumption of their residues. The European Union (EU) and the Polish government have set tolerance levels for these compounds as maximum residue limits (MRLs), which are in the range of part-per-billion.[2,3]

Carbendazim (methyl benzimidazole-2-ylcarbamate, MBC) is a systemic fungicide with protective and curative action. It is registered for use in various crops, for example, in cereal, fruit, stored fruit, and as a seed dressing.[4] Other related fungicides, e.g., benomyl and thiophanate-methyl, are degraded to carbendazim. The commercial introduction of these pesticides still leads to the need for rapid, selective, and sensitive analytical methods for the control of environmental pollution levels, especially in a staple foodstuff like cereals.

Although methods for determining pesticides in fruits, vegetables, and other complex food matrices number in the thousands (based on, i.e., gas chromatography, GC and liquid chromatography, HPLC), the pesticide residues analysis still represent an analytical challenge.[5,6]An adequate method for residue analysis should be sensitive, selective, accurate, precise, automated, cheap, applicable to a wide range of pesticides and matrices, and capable of providing unambiguous structural information. However, such perfect methods are not encountered in practice.[7]

Various analytical techniques aimed at isolating and determining the fungicide carbendazim have recently been described.[8–11] The most frequent one is the RP-HPLC technique with UV and/or fluorescence detection because carbendazim is nonvolatile and thermolabile. Nowadays, mass spectrometry (MS) coupled with GC or HPLC is the analytical technique more often used.[12,13]

The key step is the pretreatment of the sample to isolate the interesting compound from the matrix using a correct and efficient method. Over the years, several procedures have been developed with this aim, such as liquid–liquid extraction (LLE), solid phase extraction (SPE), or matrix solid phase dispersion (MSPD).

Liquid–liquid extraction is still quite popular because of the inherent simplicity, facility of operation procedure, satisfactory results, and provided by the range of organic solvents, which are available. This technique, apart from aspects, such as the labor intensity, time consuming, and the use of large volumes of often toxic organic solvents, has been used to extract some of the pesticides from different matrices.

Solid phase extraction is one of the most widely used sample preparation techniques. This method guarantees not only maintenance of the qualitative and quantitative composition of the analyzed sample but also highly reproducible analytical conditions. With this method, solvent consumption is low, the procedure is simple, and the 'life-time' of the chromatographic column is extended.

With the current trends towards miniaturization of sample preparation, several new methods have been introduced; for example, matrix solid phase dispersion. This technique offers environmentally safe extraction (essentially obviates the hazardous solvents), generates little waste, reduces the time, space, and glassware that are required for extraction. MSPD conducts the simultaneous disruption and extraction of solid and semi solid samples. The method involves the dispersal of the sample over a solid support, followed by watching and eluting with a small amount of organic solvent.

The scope of this study is to evaluate LLE, SPE, and MSPD for the extraction of carbendazim from wheat grain followed by HPLC–DAD determination. Several parameters governing the recovery of the analytes from the samples are optimized. All the three techniques were compared to establish the most suitable for quantifying the fungicide carbendazim. The method was applied to measure the levels of fungicide in wheat samples taken from the private producers.

The final determination was performed by separation using HPLC–DAD with column switching.

EXPERIMENTAL

Chemicals

Methanol was HPLC grade from J.T. Baker (Deventer, The Netherlands). Methanol, dichloromethane, phosphoric acid (H_3PO_4), hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium sulphate (Na_2SO_4), sodium phosphate dibasic (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4), were analytical grade from POCh (Gliwice, Poland). Deionized water was purified by a Maxima water purification system (ELGA, High Wycombe, England).

From these chemicals the following solutions were prepared: elution mixture (MI)–methanol/1 M HCl (83:17, v/v); elution mixture (MII)–methanol/0.1 M H_3PO_4 (1:1, v/v; pH 2); elution mixture

(MIII)–methanol/dichloromethane (1:5, v/v); phosphoric buffer pH 7; 1 M HCl; 1 M and 5 M NaOH; mobile phases—A, methanol HPLC/deionized water (45:55, v/v) and B, methanol HPLC/deionized water (60:40, v/v); injection solvent (IS)–methanol HPLC/deionized water (40:60, v/v).

Appropriate solvents were filtered through 0.45 μm Nylon 66 Membranes (Supelco, Bellefonte, PA, USA) and degassed using helium sparging.

SPE columns with Diol bed, Adsorbex 400 mg, were from Merck (Darmstadt, Germany). Silica gel (SG) was Kieselgel 60 extrapure, particle size 0.063–0.200 mm (70–230 mesh ASTM) (Merck, Darmstadt, Germany) reactivated prior to use at 773 K for 2 h and cooled in a desiccator. Acidic SG was obtained by addition of 10 mL of 1 M HCl to 100 g of SG followed by thorough mixing on a rotary mixer for 2 h. The thus prepared material was stored after equilibration in an air tight container until used. Celite 545 was from Johns-Manville Product Co. (Lompoc, CA, USA).

Carbendazim standard (purity 99.0%, Promochem, Warsaw, Poland) was used for fortification and quantitation. A stock solution of carbendazim 200 μg/mL was prepared in HPLC grade methanol. The calibration and working standard solutions of carbendazim were prepared by diluting the stock solution with methanol HPLC/deionized water (40:60, v/v). These solutions were stored in a refrigerator at 277 K.

The samples of wheat grain were collected from private producers.

Apparatus

The laboratory grain mill was type (ZBPP Bydgoszcz, Poland). The rotary vacuum evaporator was a Rotavapor-R type W (Büchi, Flail, Switzerland) with a water bath kept at 323 K. An SPE manifold was VISIPREP (Supelco), shaker was type 358S (Elpan, Lubawa, Poland). Extraction columns were packed in polypropylene cartridges 130 × 25 mm ID with a glass wool plug (Pharma-Plast A/S, Rodby, Denmark).

The HPLC system consisted of a CM3500 and a CM3200 pump, UV-DAD detector type SM 5000 set at $\lambda = 279$ nm (TSP, Riviera Beach, FL, USA); programmable, 6 port column switching valve type WEC6WK (VICI, Valco Instruments, Houston, TX, USA); 100 μL injection loop (Supelco, Bellefonte, PA, USA); Rheodyne Sample Injector Model 7125 (Rheodyne, Cotati, CA, USA). The data were collected and analyzed with an LCtalk computing system (TSP LCtalk HPLC software, version 2.03.02).

Procedures

Representative portions of wheat grain samples (200 g) was prepared using a grain mill and mixed thoroughly. Samples were extracted using the LLE, SPE, or MSPD procedure according to the scheme presented in Figure 1.

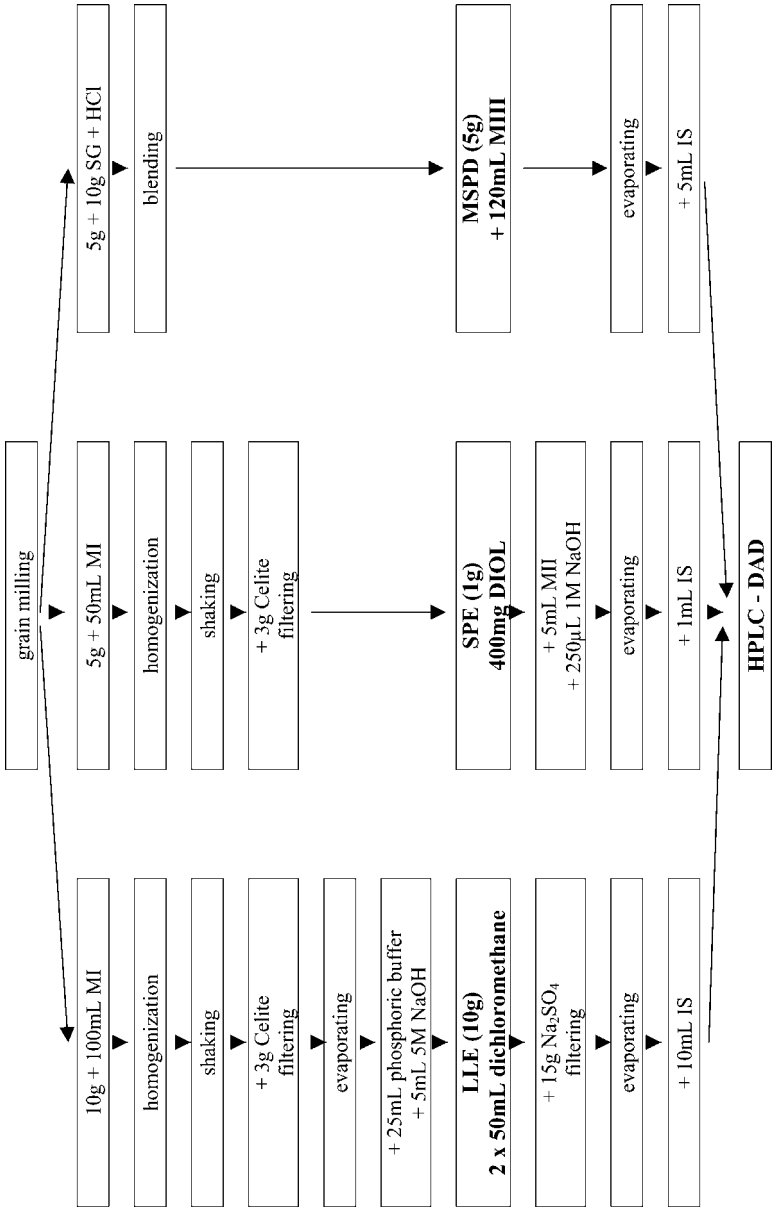

Figure 1. Scheme of the LLE, SPE, and MSPD extraction procedures for isolating and purification of carbendazim from wheat grain samples.

LLE Extraction

A 10 g portion of milled grain was sampled, 100 mL of methanol/hydrochloric acid (MI) was added, homogenized, and shaken. After vacuum filtering with additional Celite, methanol was evaporated, phosphoric buffer and sodium hydroxide were added to obtain appropriate pH, and the remaining phase was partitioned with dichloromethane. After vacuum filtering through Na_2SO_4, dichloromethane was evaporated to dryness, and the residue was redissolved in the 10 mL of the IS before injection onto the HPLC column.

SPE Extraction

A 5 g portion of milled grain was sampled, 50 mL of methanol/hydrochloric acid (MI) was added, homogenized, and shaken. After vacuum filtering with additional Celite, methanol was evaporated. The Diol extraction cartridge was conditioned by successive elution of 2 mL of methanol. The subsample extract corresponding to 1 g of grain matrix in the mixture of MI was transferred and loaded onto the SPE cartridge. The fungicide was eluted with 5 mL of MII with the addition of 250 μL 1 M NaOH. Methanol was evaporated, the extract was dissolved in 1 mL of the IS, and was injected into the chromatographic system.

MSPD Extraction

A subsample of 5 g of milled grain was weighed into a mortar of ca. 10 cm diameter, 10 g of acidic SG was added and ground to obtain a homogeneous mixture. The extraction column was fitted with a polyethylene frit, the powdery sample was transferred through a widemouth polypropylene funnel (10 cm top ID). Mortar and pestle were rinsed with 20 mL of MIII, and the rinsings were carefully poured into the column. The carbendazim residues were extracted with total volume of 120 mL eluent and collected in round-bottomed flasks. The solvent was evaporated to dryness using a rotary evaporator, and the dry residue was dissolved in 5 mL of the IS before the injection onto the HPLC column.

HPLC Analysis

Extracts of the fungicide carbendazim from grain samples were analyzed in an isocratic HPLC column switching system equipped with the clean up column, Supelcosil LC-8-DB, 150 × 4.6 mm ID 5 μm (Supelco Inc., Bellefonte, PA, USA), and the analytical column Alltima C18, 250 × 4.6 mm ID, 5 μm (Alltech, Carnforth, UK). The separation columns were kept at ambient temperature. Mobile phases were: A, methanol/deionized water (45:55, v/v) and B, methanol/deionized water (60:40, v/v). The DAD detector was set

at 279 nm. Flow rates for both pumps were 1 mL/min; injection volume was 100 μL. The column switching procedure was described in details in reference of Michel et al.[14] and Michel and Buszewski.[15]

Method Validation

All validation procedures were performed using pesticide free wheat grains. The percentage of recovery rate (RR) and the precision of extraction techniques were determined at three spiked levels 0.04, 0.08, and 0.1 μg/g by spiking with the working solutions of carbendazim. The spiked samples were allowed to stand overnight before extraction. Five replicated samples of each three spiked levels were extracted and analyzed. Control samples, without being fortified with carbendazim, and blank samples, without grain matrix, were also prepared. RR data were determined by comparing the analyte concentration after extraction of spiked samples

$$RR = \frac{c}{c_0} \cdot 100\% \tag{1}$$

where: c–analyte concentration; c_0–standard concentration; and to ensure that the method would perform satisfactorily for a wide range of residue amounts, from detection to maximum residue limits.[16] All the samples were analyzed consecutively in the same day, for the same analyst to study repeatability. Precision was measured by the relative standard deviation (RSD) of the set of n repeat measurements and is defined as:[17]

$$RSD = \frac{s}{\bar{x}} \tag{2}$$

where: s–standard deviation

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}} \tag{3}$$

$\bar{x}$–mean (average) measurement; x_i–single measurement.

The limits of quantification (LOQ) were determined as the lowest concentration of a given pesticide, giving a response that could be quantified with RSD of less than 20%.

RESULTS AND DISCUSSION

The treatment of crops with pesticides makes these compounds to be deposited on the surface of plants, in the aerial treatments, or to be absorbed through the roots when applied to soil. In general, for cereal plants, pesticides are often found at higher concentrations in straw than in grains. This fact makes the expected pesticide levels in cereal grains to be low, which increases the

difficulty of analysis. Therefore, analytical methods should be highly sensitive and selective to allow the determination of pesticides in those matrices, and, consequently, the extraction and clean up of extracts are important steps of the analytical procedure.

Optimization of Analytical Procedures

Extraction of the benzimidazole fungicide carbendazim from wheat grain samples was optimized by means of studying the parameters that influence this process. To determine extraction efficiency, control samples were fortified with pesticide stock solutions. Recovery studies were made, so as to reproduce as far as possible, the natural incorporation of the residue in a sample matrix. Physical binding and the formation of conjugates is more likely in grains, therefore, a very through mixing with the spiking solution, followed by standing overnight for equilibration was done. Recoveries were calculated from five replicates of each sample.

Classical analytical methodology is based on liquid-liquid extraction, whereas modern techniques have been developed based on solid-liquid extraction. As a classical extraction technique, LLE was compared with two other, SPE and MSPD techniques. The LLE technique of isolating carbendazim from cereal material was developed nearly eight years ago on the basis of the study[9] and is still in routine analysis. The analytes to be extracted are partitioned between two immiscible liquids, rather then between a solid and a liquid, as in SPE where the analyte must have a greater affinity for the solid phase than for the sample matrix (retention or adsorption step). Compounds retained on the solid phase can be removed at a later stage by eluting with a solvent with a greater affinity for the analytes (elution or desorption step).[18,19] In the investigations, the isolation of carbendazim by SPE from wheat grain were conducted due on earlier works,[20,21] and according to guidelines from literature.[22,23]The procedure was modified on the needs of the investigations. The volume of elution mixture MII was selected and set up at 5 mL during the optimization steps.

The idea of separation mechanisms, which rule in the case of solid-phase extraction, was the basis to elaborate on the practical technique of sample preparation–MSPD.[24–27] It has been demonstrated that mixing biological samples with silica supports promotes disruption of the sample structure by the mechanical blending. As a difference with SPE, where much of the sample is retained in the first millimeters of the column, the sample is dispersed throughout the length of the column in MSPD. The new phase, together with the pesticide distribution and its interactions, allow specific solvent elution of the compound of interest and these are the main controlling factors of this sample preparation technique. More detailed descriptions of the basic principles of this modern sample preparation technique for the extraction of plant materials are available in a number of excellent review articles, which

recently appeared in the literature.[28–30] The optimization of the MSPD procedure for the isolation of carbendazim from wheat grain is presented in the author's publication.[31]

Method Comparison

The procedures involving LLE, SPE, and MSPD extractions were validated for wheat grain samples fortified at levels 0.04, 0.08, and 0.1 μg/g. Therefore, ground cereal samples were spiked with adequate working standard solution volumes prior to extraction. Up to five replicate analyses were run at all fortification levels and extraction techniques. Figure 2 shows the average percentage of recovery values and RSDs. The average recoveries for LLE were between 71.2–76.5%, for SPE 82.2–83.2%, and for MSPD 84.3–90.7%. RSDs ranged from 5.2% for LLE, 3.1–4.6% for SPE, and 2.7–4.1% for MSPD.

The limit of quantification (LOQ) at $\lambda = 279$ nm was 0.02 μg/g for all extractions. This value is lower than the Polish tolerance limit for carbendazim in cereals (0.1 μg/g). According to EU guidelines,[16] the mean recoveries at each fortification level should be in the range of 70–110%. The LOQ was defined as the lowest level for which acceptable recoveries and repeatabilities (<20%) are obtained. Table 1 summarizes several parameters indicative of the analytical performance of the three methodologies described.

The results show that the three present procedures work well at all levels. The average recoveries for LLE were the lowest, but values of RSD were identical for both SPE and MSPD. The time required for extraction was the lowest when MSPD was used, and the highest for LLE. Use of disposable and inexpensive equipment was the lowest when MSPD is used and was

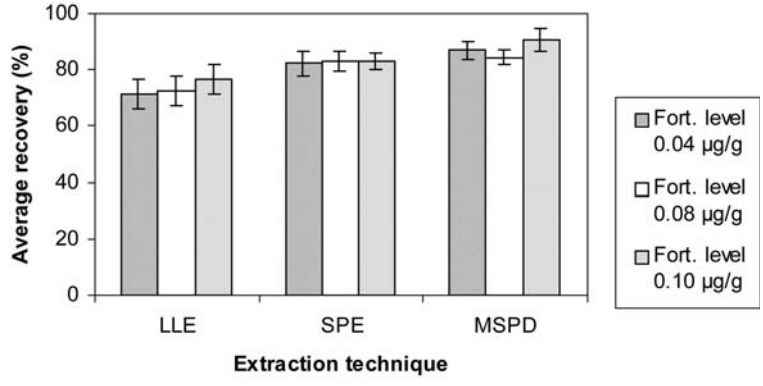

Figure 2. The comparison of recovery rates (RR) and relative standard deviations (RSD) of carbendazim residue obtained from optimization parameters of LLE, SPE, and MSPD extraction techniques.

Table 1. Method performance comparison

	LLE	SPE	MSPD
Spiking concentration (μg/g)	0.04–0.1		
Accuracy (% recovery)	71.2–76.5	82.2–83.2	84.3–90.7
Repeatability (% RSD)	5.2	3.1–4.6	2.7–4.1
Linearity (R^2)	0.9997		
Sensitivity (LOQ, μg/g)	0.02		

more expensive for SPE. Consumption of organic solvents: minor amounts of it are used with MSPD compared to SPE and LLE, which involves large amounts of dichloromethane. Facility of operation was superior for MSPD. The possibility for automation was highest for SPE, and next for MSPD. Several other parameters are: emulsion formation can cause problems during LLE, less time and less organic solvents are needed for cleaning glass equipment because disposable SPE cartridges and MSPD columns are used for extraction.

Figure 3 displays the chromatograms of the fortified subsamples of wheat grain on the clean-up column coupled directly to UV-DAD detector and extracted by LLE, SPE, and MSPD extraction techniques Differences in sensitivity between the three extraction methods can be clearly observed in this figure.

Summarizing, the high efficiency of MSPD can be clearly observed by comparing the recovery data with those obtained with LLE and SPE. In order to obtain the highest efficiency, MSPD is the method of choice. The results showed good performance of the analytical protocol with wheat grain samples.

HPLC Analysis

Our proposed HPLC procedure allowed direct determination without derivatization, with no buffer conditions, less co-extractive interference, and satisfactory low quantification limits.

The calibration curve was obtained by plotting peak height (in LCtalk units) versus concentration of carbendazim (μg/mL) over the range from 0.10 to 3.20 μg/mL with UV detection set at 279 nm for 100 μL injection. The straight line obtained corresponds to the equation

$$y = 201092x + 10556 \tag{4}$$

and is presented in Figure 4. The coefficient of correlation was $R^2 = 0.9997$.

The relevant aspects of applying column switching in our study were to increase chromatographic selectivity and sensitivity, to enrich trace amounts in the sample, to protect the UV-DAD detector, and to speed up the column

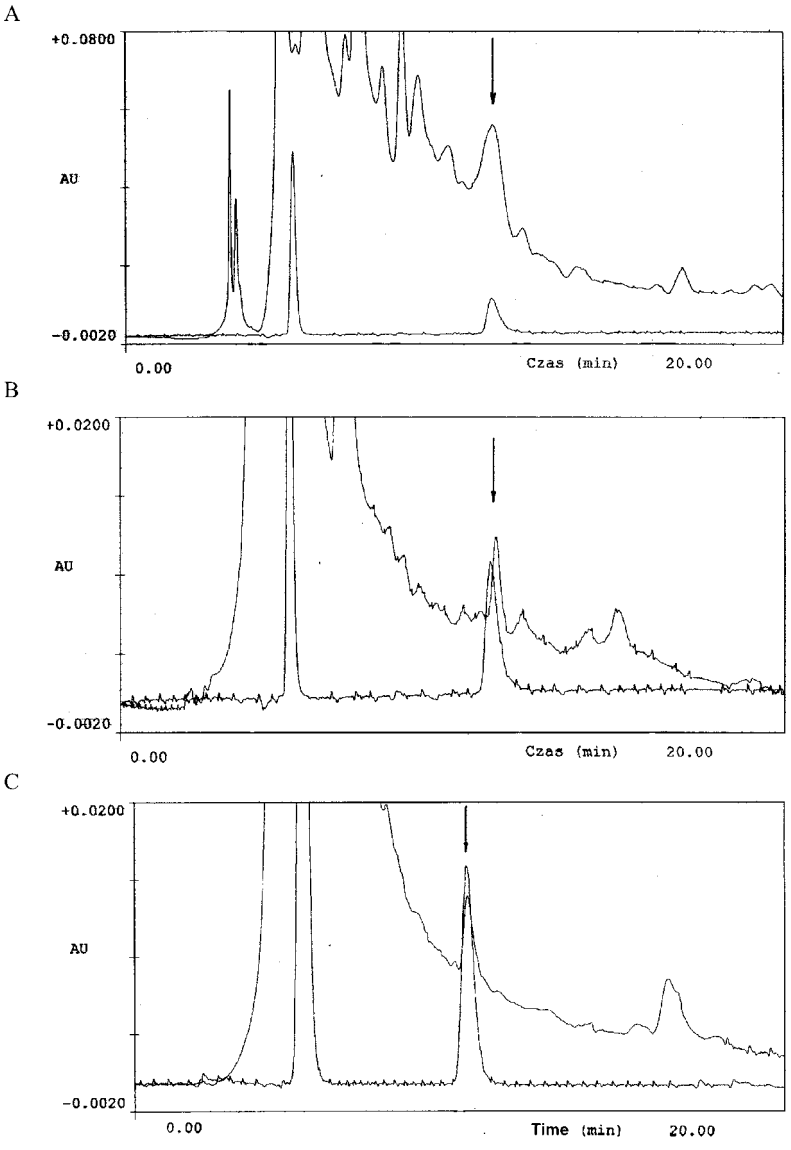

Figure 3. Chromatograms of 5 g fortified subsamples of wheat grain on clean-up column coupled directly to UV-DAD detector after A: LLE extraction; B: SPE extraction; C: MSPD extraction. Arrows indicate carbendazim.

stabilization. The possibility of performing an automated and efficient clean up of extract samples is a highly desirable option in analysis. Figure 5 illustrates typical chromatograms of the carbendazim standard, unfortified and fortified wheat grain sample extracts using the MSPD technique. No

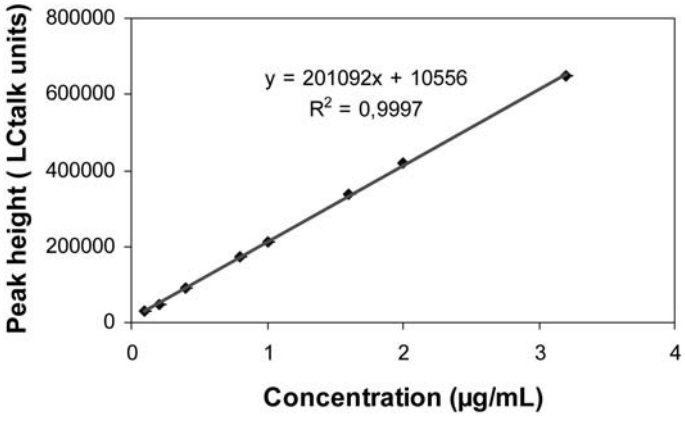

Figure 4. Calibration curve for carbendazim.

interfering peaks were observed on the chromatogram of the unspiked extracts obtained under the selected conditions.

Application to Real Samples

The three procedures were verified by analyzing 186 wheat grain samples taken from private producers located near Toruń city. The results of samples are given in Table 2.

It is interesting to note the good agreement between the results obtained by the three procedures (data not presented) for samples with carbendazim residue above MRL. The fungicide carbendazim residue concentrations found in wheat grain were near 97% lower than the limits established by the EU or the Polish legislation, which demonstrated the good quality of the Polish wheat for human consumption.[3]

CONCLUSION

HPLC–DAD with column switching determination provided sensitive and selective identification and quantitation of carbendazim. It can be successfully combined with the state-of-art extraction procedures to be applied for monitoring control of wheat grain.

The consumption of wheat cereal is clearly at a significant level, but not too many analytical procedures have been reported for determining pesticide residues in these matrices. Due to the increasing public concern of the presence of pesticides in food commodities, analytical methods must be developed.

The use of classical extraction techniques requiring large volumes of harmful solvents has been overcome by new techniques based on the

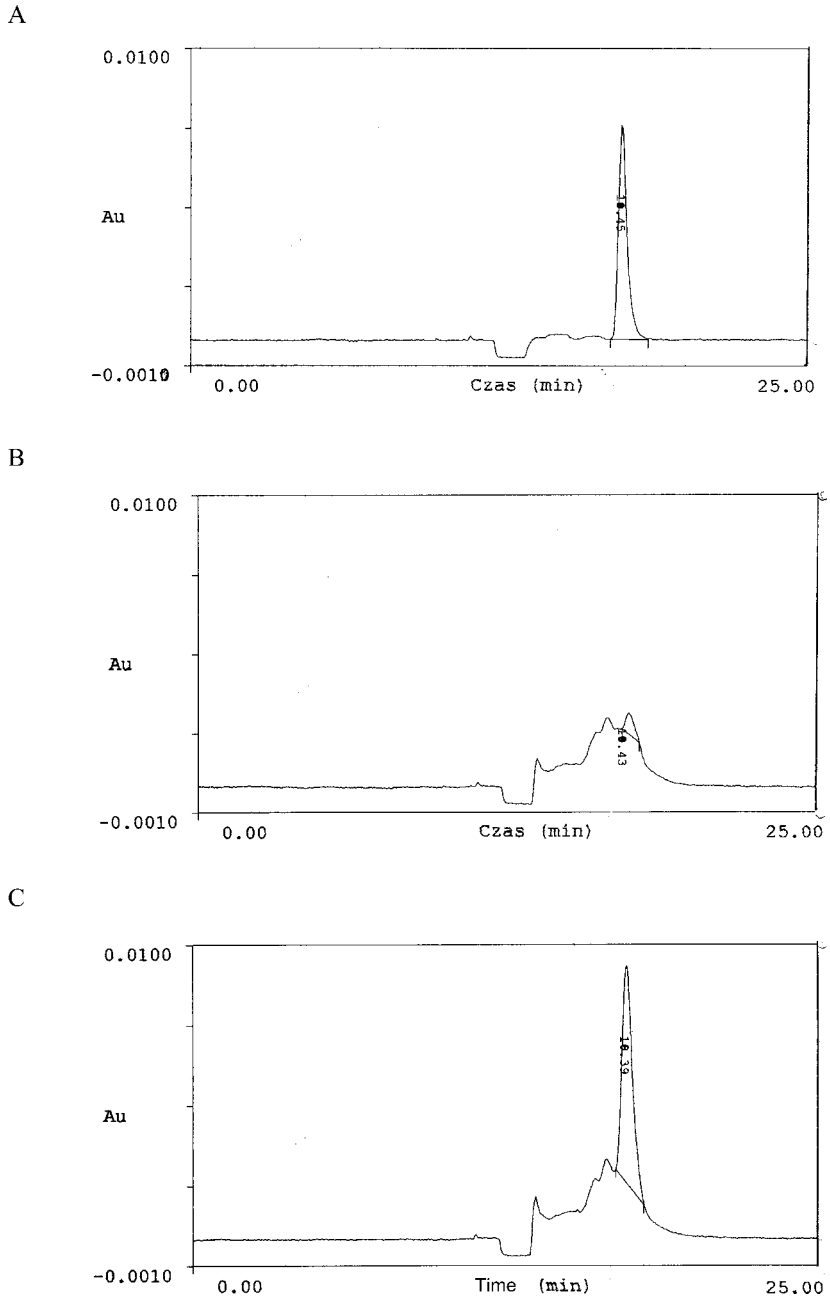

Figure 5. A: chromatogram of carbendazim standard 0.4 μg/mL (R_t = 18.45 min); B: chromatogram of a 5 g subsample control wheat grain (non-fortified); C: chromatogram of a 5 g subsample control wheat grain fortified at 0.08 μg/g (84.3% recovery).

Table 2. Carbendazim residue concentrations in wheat grain from private producers

Pesticide	Carbendazim
No of samples	186
No of samples without residue	150 (80.7%)
LOQ (μg/g)	0.02
No of samples with residue (μg/g)	
0.02–0.049	16 (8.6%)
0.05–0.079	9 (4.8%)
≥0.08	5 (2.7%)
MRL (μg/g)	0.1
No of samples with residue above MRL	6 (3.2%)

LOQ: limit of quantification.
MRL: maximum residue level.

solid-phase extraction of pesticides. Sample preparation using solid-phase extraction followed by the determination of carbendazim residue by a chromatographic method provides rapid, reliable, and sensitive procedures for the analysis of pesticide at levels usually found in food. The reported levels are generally very low and, therefore, they represent a low consumer exposure to pesticides through the consumption of food commodities.

However, the results presented in this report indicate that MSPD is an excellent extraction technique for preconcentrating the fungicide carbendazim. The main advantage of the described extraction methods compared with a traditional method is the higher accuracy, precision, and sensitivity, but also the low cost of a unit and significant reduction of the required volume of organic solvent.

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