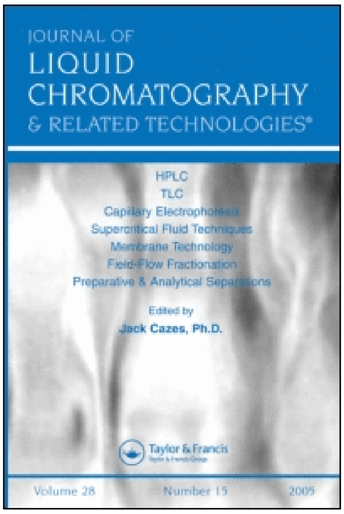

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HPLC Determination of Pesticides in Soybeans using Matrix Solid Phase Dispersion

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Abstract: A method to determine six pesticides applied in soy cultivation (imazethapyr, imazaquin, metsulfuron-methyl, carboxin, chlorimuron-ethyl, and tebuconazole) using matrix solid phase dispersion (MSPD) as the extraction technique followed by a clean-up step, using a C_8 co-column, and subsequent chromatographic analysis by high performance liquid chromatography diode array detection (HPLC-DAD) was developed. The validated method showed good recuperation for all pesticides (60–120%), except for metsulfuron-methyl and tebuconazole at their lowest concentration levels, and the quantification limits of the method (0.04–0.08 $\mu g\ g^{-1}$) were below the maximum residue limits imposed by the principal regulatory agencies.

Keywords: Soy, Multiclass pesticides analysis, Sample preparation, MSPD, Method validation, HPLC-DAD

INTRODUCTION

In the evolution of agriculture, pesticides have become an important tool for plant protection, increasing the quantity and quality of the food. However, the continuous and even expansive application of pesticides leaves residues,

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contaminates waters, soil, and food, and consequently, compromises human health and pollutes the environmental.

Expansion of soy cultivation is associated with agricultural growth, and soybeans are much used as a foodstuff because of their functional properties: rich in vitamins, proteins, lipids, and minerals.[1–4] It is a cultivation that gives high yields and is easily adapted to most places in the world.[4,5] However, the use of pesticides is necessary to guarantee production. On the other hand, they compromise human health because of their high toxicities.[6,7] To preserve human heath, the monitoring of the pesticides used in soy cultivation is necessary, however, only a few methodologies have been developed because of the difficulty in extracting the pesticides from a fatty matrix such as soybeans.[8]

The analysis of residues of pesticides on foods requires use of sophisticated extraction techniques, due to the low maximum limits of pesticides residues (MRL) imposed by the regulatory agencies and mainly due to the complexity of these matrices. Recently, for extraction of pesticides from solid or semi-solid samples, modern extraction techniques, such as matrix solid phase dispersion (MSPD), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), and microwave assisted extraction (MAE) have been shown to be faster, require less organic solvents, fewer clean-up steps, and provide cleaner extracts than are obtained with classical methods.[9,10]

MSPD is a technique which involves the dispersion of the sample over a solid support, and subsequent elution of the compounds with a relatively small volume of solvent. Moreover, extraction and clean-up may be performed in the same step, reducing the analysis time and solvent employed.[11–14] This extraction technique has been applied to other fatty content matrices, such as okra,[15] olives and olive oil,[16] almonds,[17] and honey.[10]

In this work, an extraction procedure based on MSPD was developed for determination of the most common pesticides applied to soybean cultivations in Brazil: imazethapyr and imazaquin (imidazolinone herbicides), metsulfuron-methyl (triazinylsulfonylurea herbicides), carboxin (anilide fungicides), chlorimuron-ethyl (pyrimidinylsulfonylurea herbicides), and tebuconazole (conazole fungicides). This was followed by HPLC-DAD analysis. The method was validated in agreement with the International Conference on Harmonization (ICH)[18] and the Nacional Institute of Metrology, Standardization, and Industrial Quality (INMETRO).[19] Subsequently pesticide residues in soybean samples were analyzed.

EXPERIMENTAL

Chemicals and Materials

The pesticide standards: imazethapyr (99%), imazaquin (99%), metsulfuron-methyl (98%), carboxin (99%), chlorimuron-ethyl (99%), and tebuconazole (98%) were all purchased from Chem Service (West Chester, PA, USA).

The solvents for sample preparation, pesticide grade ethyl acetate, and chromatographic analysis, HPLC grade acetonitrile, HPLC grade methanol, were purchased from Tedia (Rio de Janeiro, Brazil) and phosphoric acid was obtained from Mallinckrodt (Xalostoc, Mexico). Deionized water was obtained from a Milli-Q Plus system from Millipore (Bedford, MA, USA). All solvents were filtered using a 0.45 μm poly(vinylidene)difluoride (PVDF) membrane from Millipore (São Paulo, Brazil).

The sorbents used in the extraction procedure, silica (35–70 μm) and Florisil (70–150 μm), were purchased from Acros (Geel, Belgium); while C_{18} and C_8 (45 μm) were purchased from Supelco (Bellafonte, PA, USA).

Standard Preparation

A stock standard solution of each pesticide was prepared by dissolving 10 mg of each compound in 10 mL of acetonitrile and stored in a refrigerator at 4°C. These solutions were stable for at least four months. The working solutions used to construct the analytical curves and fortify the samples were prepared in acetonitrile over a concentration range from 0.08 to 2.4 $\mu g\ g^{-1}$ and these solutions were stable for at least one week.

Chromatographic Instrumentation and Conditions

The HPLC system consisted of two Waters 515 pumps with a gradient programmer, a 7725i Rheodyne (Cotati, CA, USA) injector with a 10 μL loop, and a Waters 996 photodiode array detector. Data acquisition and treatment was performed by Millenium[32] v. 3.05. A Phenomenex Synergi™ Fusion-RP C18 column (150 mm × 4.6 mm i.d.), having an embedded polar group and 4 μm particles, and a similar guard column (4 mm × 3 mm i.d.), were used for the separations. The mobile phase was acetonitrile:water (acidified to pH 3.0 with phosphoric acid) and the gradient, used at a flow rate of 0.5 $mL\ min^{-1}$, was: 0 min, 30:70 v/v; 10 min, 40:60 v/v; 13 min, 55:45 v/v; 15 min, 70:30 v/v; 17 min, 70:30 v/v; 18 min, 60:40 v/v; 20 min, 50:50 v/v; 22 min, 30:70 v/v; 28 min, 30:70 v/v. Detection was at 221 nm. The quantification of the pesticides was at the highest absorption wavelength of each pesticide (imazethapyr, carboxin, and chlorimuron-ethyl at 215 nm; imazaquin at 254 nm; metsulfuron-methyl at 225 nm, and tebuconazole at 221 nm). All separations were carried out at room temperature.

Sample Preparation

Pesticide-free soybean samples (Mãe Terra) were obtained from a Pão de Açúcar supermarket (Campinas, São Paulo, Brazil). Real soybean samples were obtained from Cooperativa Tritícola Taperense, Ltda. (Tapera, Rio Grande do Sul, Brazil), and were of two different varieties, CD-205 and CD-213.

The samples were homogenized with a liquefier (Britânia) during 5 minutes for obtaining small particles and than used in the extraction procedure.

MSPD Extraction

One gram of soybeans was placed in a glass mortar and gently blended with 2 g of silica (dried at 140°C for 12 h) for 10 min using a pestle. This mixture was introduced into 6 mL poly(propylene) syringes (Supelco) retaining it with two polyethylene frits (20 μm pore size, Supelco) already containing 0.5 g of co-adsorbent. The syringes were connected to a 12 port SPE vacuum manifold (Supelco) adjusting the flow to 1 mL min^{-1}. After 30 minutes, the pesticides were eluted, with 4 portions of 5 mL each of ethyl acetate and 1 portion of 5 mL of methanol. The eluent was concentrated to dryness with a slow flow of dry nitrogen and reconstituted in 0.5 mL of acetonitrile. Then, the sample was filtered through a 0.45 μm Millex (Millipore) filter and 10 μL were injected in the chromatographic system.

Method Validation

A validation procedure, according to the ICH[18] and INMETRO,[19] was conducted to determine response linearity, recoveries of the pesticides, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision (repeatability and intermediate precision).

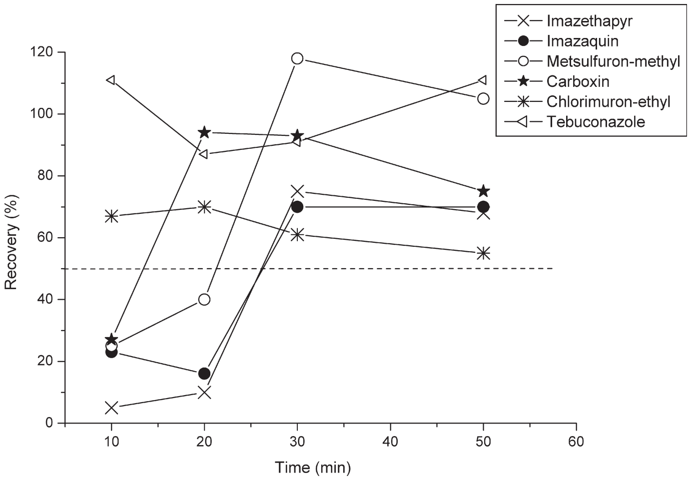

Figure 1. Variation of the percent recoveries with the interaction time of the pesticides with silica, after the dispersion step.

The parameters, LOD and LOQ, were determined based on signal to noise ratios of 3 and 10, respectively, using individual injections. The analytical curves were made in matrix matched solutions using six different concentrations (0.08–2.4 μg g^{-1}) for each pesticide, which was added to the extracts after the extraction procedure with the soybeans, with three replicates each. The linearity was estimated using linear regression analysis by the least square regression method and the linear ranges were obtained from the analytical curves.

The accuracy was determined as percent recovery at three different fortification levels (1x, 2x, and 10x LOQ). Precision was evaluated in terms of repeatability at three different fortification levels, with three replicates at each level, and intermediate precision was calculated with three replicates at each of three concentration levels on three different days. The numerical values of precision were expressed by coefficient of variation (CV) of triplicate measurements of the analytes, using the equation:

$$CV = 100\frac{s}{x_m}$$

where

$$s = \left[\sum (x_i - x_m)^2/(n-1)\right]^{1/2}, \quad x_m = \sum x_i/n, n$$

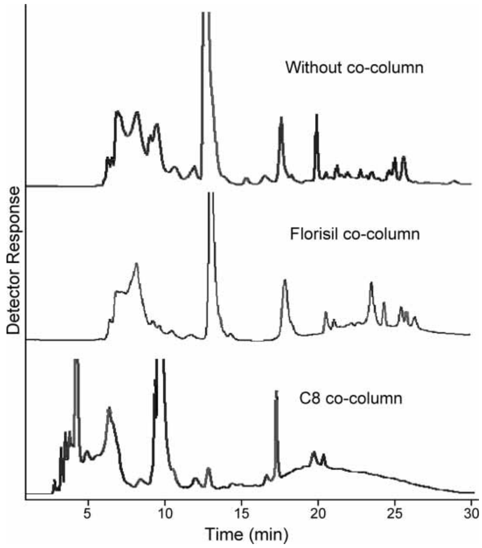

Figure 2. Chromatograms obtained with and without Florisil or C_8 co-columns. Chromatographic conditions: mobile phase: acetonitrile:water in the gradient elution mode (see Experimental); flow rate: 0.5 mL min^{-1}; injection volume: 10 μL; detection: UV at 221 nm.

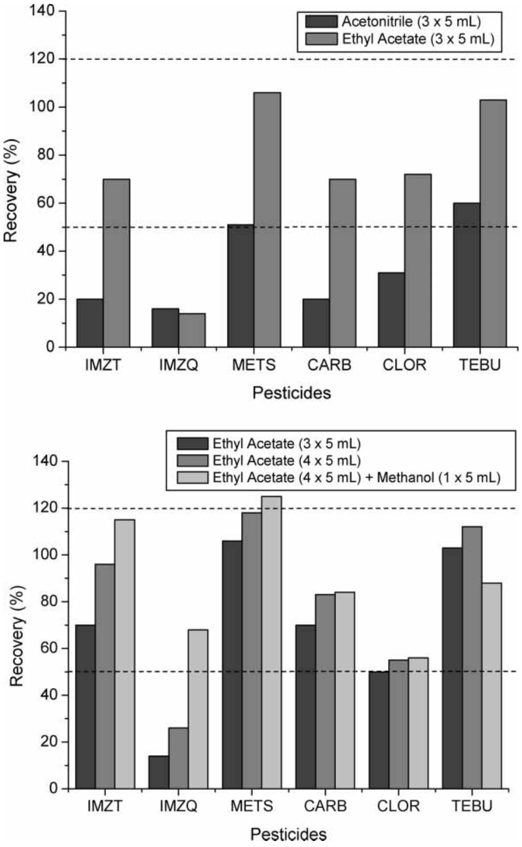

Figure 3. Recoveries of the pesticides: imazethapyr (IMZT), imazaquin (IMZQ), metsulfuron-methyl (METS), carboxin (CARB), chlorimuron-ethyl (CLOR), and tebuconazole (TEBU) with different elution solvents ($n = 2$), during the optimization step. Samples fortified with 2 × LOQ of each pesticide.

is the total number of measurements, and x_i is value of the individual measurement.

RESULTS AND DISCUSSION

Extraction Procedure

Different parameters that affect MSPD extraction, such as dispersion sorbent, interaction time after dispersion, clean-up, and elution solvents were studied. The dispersion sorbent was chosen based on the polarity of the compounds because the interactions between the sorbent and the compounds depend mainly on their relative polarities. So, the sorbent chosen for this work was

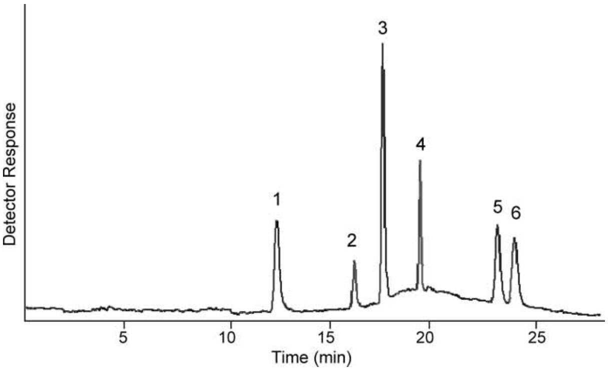

Figure 4. Chromatogram showing separation of the pesticides. Chromatographic conditions: mobile phase: acetonitrile:water in the gradient elution mode (see Experimental); solution concentration: 10 μg g^{-1}; flow rate: 0.5 mL min^{-1}; injection volume: 10 μL; detection: UV at 221 nm. Identification: (1) Imazethapyr, (2) Imazaquin, (3) Metsulfuron-methyl, (4) Carboxin, (5) Chlorimuron-ethyl, and (6) Tebuconazole.

silica activated at 140°C for 12 h. C_{18} also was tested, but the compounds presented little or no retention on this sorbent. After the dispersion step, the time necessary to complete the migration of the pesticides to the silica was evaluated. Times from 10 to 50 min were tested, and the results showed that 30 min was sufficient for complete migration of all the pesticides (Figure 1).

Due to the complexity of the matrix, high fat content, and high percent of proteins, the extracts obtained presented elevated quantities of impurities, so a clean-up step was necessary. Two sorbents with different physical and chemical characteristics, Florisil and C_8, were tested. The results, comparing the chromatograms (Figure 2), showed that Florisil eliminated only a few interferents, and that C_8 was more efficient in removing a greater number of interferents.

Several elution solvents, including acetonitrile, ethyl acetate, and methanol, and the volume of each, were evaluated for extraction of the pesticides from the samples. The results are shown in Figure 3. Ethyl acetate was more efficient than acetonitrile, but was inefficient for eluting imazaquin. An increase of the eluent volume improved the recuperation values for all the pesticides, but was still ineffective for imazaquin. Therefore, a final portion of a solvent more polar than ethyl acetate, such as methanol, was tested. This was efficient and increased the recuperation values of imazaquin by approximately 30%. Thus, the elution solvents were defined as four portions of 5 mL each of ethyl acetate and one portion of 5 mL of methanol.

Method Validation

With the experimental conditions for MSPD extraction established, the method was validated. Figure 4 shows a chromatogram of the pesticides

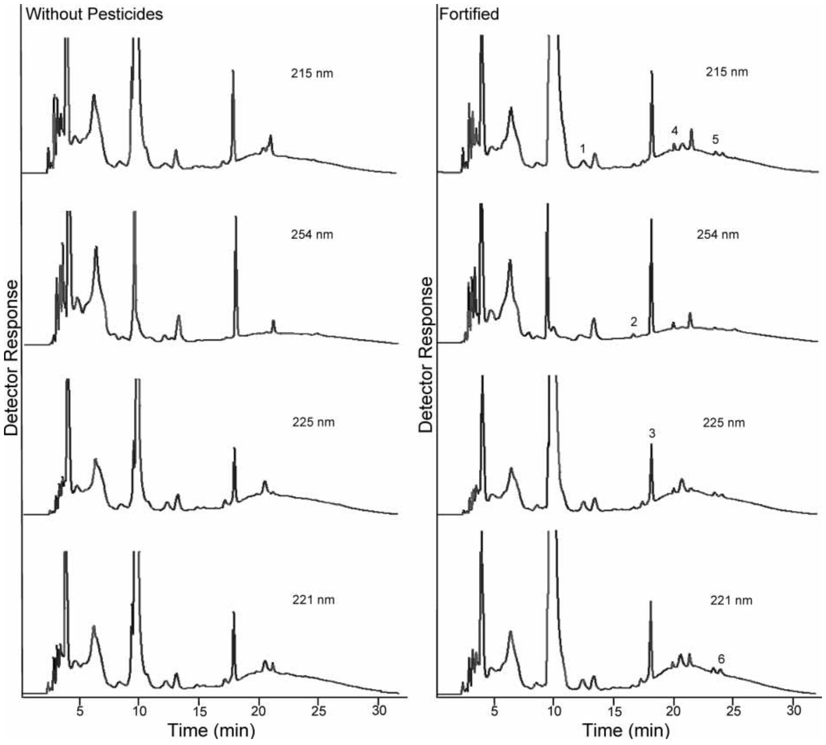

Figure 5. Chromatograms of the extracts from soybeans samples without pesticides and samples fortified with a standard pesticide solution at 2 μg g^{-1}, obtained after MSPD methodology optimization. Chromatographic conditions and peak identifications as in Figure 4.

separation and Figure 5 shows chromatograms obtained for a soybeans sample without pesticides and samples fortified with a standard pesticide solution at 2 μg g^{-1}. The chromatograms showed that impurities remained with the same retention times as imazethapyr and metsulfuron-methyl, compromising the quantification of these pesticides at the lowest concentrations levels.

The parameters of the analytical curves are presented in Table 1. Good linearity and correlation coefficients greater then 0.97, without visible bias, were obtained. The results of LOD and LOQ, before and after concentration, are presented in Table 2. The LOQ values obtained for all pesticides, after concentration, are below the tolerance levels for these pesticides in soybeans.[20]

The results of accuracy, repeatability, and intermediate precision are presented in Table 3. The results of accuracy, considered as acceptable recoveries between 70 and 130%,[21] were satisfactory for all pesticides, except for imazethapyr, metsulfuron-methyl, and tebuconazole at the lowest concentration level. The repeatabilities and intermediate precision results were

Table 1. Analytical curve parameters

Pesticide	Analytical curve a (intercept)	 b (slope)	 r	Linearity (μg g^{-1})
Imazethapyr	2574	53	0.970	0.08–1.200
Imazaquin	−840	61	0.984	0.100 –1.500
Metsulfuron-methyl	−3604	68	0.982	0.140–2.100
Carboxin	586	46	0.998	0.140–2.100
Chlorimuron-ethyl	51	21	0.995	0.100–1.500
Tebuconazole	−3385	35	0.997	0.160–2.400

$y = a + bx$; a = intercept; b = slope; r = correlation coefficient.

Table 2. Limits of detection (LOD) and limits of quantification (LOQ) of the pesticides extracted from soybeans by MSPD and MRL[a]

Pesticide	LOD[b] (μg g^{-1})	LOQ[b] (μg g^{-1})	LOD[c] (μg g^{-1})	LOQ[c] (μg g^{-1})	MRL[a] (μg g^{-1})
Imazethapyr	0.025	0.080	0.012	0.040	0.100
Imazaquin	0.032	0.100	0.016	0.050	0.050
Metsulfuron-methyl	0.040	0.140	0.020	0.070	—
Carboxin	0.040	0.140	0.020	0.070	0.020
Chlorimuron-ethyl	0.030	0.100	0.015	0.050	0.050
Tebuconazole	0.046	0.160	0.023	0.080	0.100

[a] ANVISA.[20]

[b] Without concentration (instrument values).

[c] After 2 times concentration.

Table 3. Recoveries and precision (repeatability and intermediate precision), expressed as % CV, for pesticides in soybean samples ($n = 3$)

	Recovery (%)			Repeatability (% CV)			Intermediate precision (% CV)		
Pesticide	F1	F2	F3	F1	F2	F3	F1	F2	F3
Imazethapyr	60	117	118	24	27	17	24	26	15
Imazaquin	85	89	81	18	6	5	8	7	8
Metsulfuron-methyl	231	114	98	8	25	8	14	24	14
Carboxin	110	108	115	4	15	3	8	7	8
Chlorimuron-ethyl	77	68	100	17	3	14	11	16	13
Tebuconazole	190	120	110	5	9	5	5	9	8

F1 = 1 × LOQ; F2 = 2 × LOQ e F3 = 10 × LOQ.

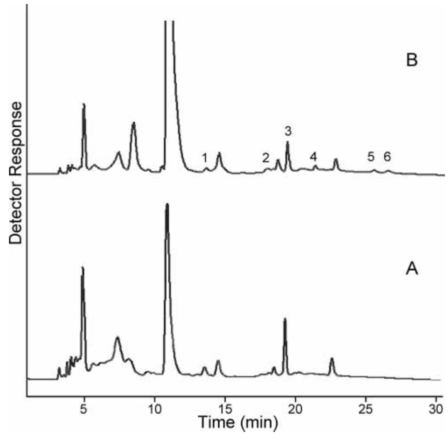

Figure 6. Chromatograms of a soybean samples (CD-205) (A) without and (B) fortified with a standard pesticides solution at 3 μg g^{-1}. Chromatographic conditions and peak identifications as in Figure 4.

satisfactory for all pesticides (3–18%) except for imazethapyr and metsulfuron-methyl at the lowest concentration levels (24–27%), where acceptable values are considered up to 20%.[21]

Analysis of Pesticide Residues in Soybeans Samples

To evaluate the effectiveness of the proposed method, it was applied to the analysis of two soybean samples (CD-205 and CD-213). None of the pesticides were detected in these tests. To confirm the efficiency of the proposed method, it was also applied to the analysis of a fortified sample of these commercial soybean samples, with a standard pesticide solution at 3 μg g^{-1}. Representative chromatograms of these samples are shown in Figure 6.

CONCLUSIONS

The proposed extraction method, MSPD, is a suitable extraction procedure for solid and complex matrices, as is the case of soybeans, and is a rapid and simple technique. Pesticide migration from soybean to silica is probably facilitated by the high fat content. However, it requires the optimization of some variables during the process of methodology development. A clean-up step was necessary because of the amount of matrix impurities, but the majority was eliminated using a C_8 co-column. Remaining impurities with the same retention time as imazethapyr and metsulfuron-methyl do not invalidate the determination, but compromise the quantification of the compounds.

The results of method validation demonstrate that the proposed methodology presented good accuracy and precision, limits of detection and quantification that allowed determinations below the maximum residues limits[20] for the pesticides (imazethapyr, imazaquin, metsulfuron-methyl, carboxin, chlorimuron-ethyl, and tebuconazole) in soybean samples using HPLC-DAD.

Application of the method developed for analysis of pesticide residues in soybean samples confirmed the efficiency of the method.

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