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ARTICLE

Development of a Rapid Determination of Pesticides in Coated Seeds Using a High-Performance Liquid Chromatography−UV Detection System

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For the determination of pesticides in coated seeds, this study compared two HPLC−UV methods, using a short column or a conventional column, as well as two extraction procedures, by ultrasonic extraction at room temperature or by pressurized liquid extraction (PLE). The comparison of selected column performances showed that the short column enabled the 3-fold reduction of analysis time (ca. 9 min vs 29 min) and eluent consumption (ca. 6.1 mL vs 20.8 mL) for the separation of five insecticides (bitertanol, fludioxonil, imidacloprid, metalaxyl-M and tefluthrin) and one bird repellent (anthraquinone) without altering peak resolutions. Recovery rates for pressurized liquid extraction at 120 °C were similar (between 84% and 102%) to those obtained by ultrasonication. Both methods were then applied for the extraction of loaded seeds. Rates for ultrasonic extraction at room temperature were lower (from 16% to 95%) than those observed for recovery tests, unlike PLE at 120 °C which showed good rates, ranging between 82% and 95%, for all the loaded pesticides.

KEYWORDS: Pesticides; seed; coating; liquid chromatography; short column; pressurized liquid extraction

INTRODUCTION

From the harvest to the culture, through storage and sowing, seeds are constantly threatened by fungi (such as *Fusarium* (*1*) and *Pythium* (*2*, *3*)), diseases (like smut (*4*)), insects (such as wireworms (*5*)), rodents or birds. Thus, seeds are currently treated with water-based flowable concentrates, which are generally a combination of insecticides, fungicides and repellents.

For quality control, seed producers have to determine the distribution of pesticides in coated seeds. At the same time, because of the public concern about pesticide in foodstuffs, modern analytical methods have been developed to quantify pesticide residues regarding expenses, preparation time and ability to extract the widest range of pesticides.

Pesticide residues on fruits, vegetables and cereals are generally extracted by liquid extraction with acetonitrile, acetone or ethyl acetate (*6*−*9*). Extracts are purified or not by partitioning (*10*, *11*) and/or solid phase extraction (*7*, *9*). In 2004, Granby et al. (*12*) developed a pesticide residue extraction method using a methanol solution assisted by ultrasonication. Though a lot of publications dealt with the determination of pesticide residues on seeds, only Schlatter (*13*) and Huijbregts (*14*) reported the determination of pesticides on individual loaded seeds by HPLC. In his work, Schlatter extracted pesticides by sonicating one single seed in a mixture of deionized water−acetonitrile in order to evaluate the seed-to-seed distribution of loaded pesticides, but no work has been realized about the determination of pesticides on a larger amount of loaded seeds. For the past decade, pressurized liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction) has appeared as a promising sample preparation procedure, presenting the advantage of being automated. It can be used either on wet samples (such as fruits and vegetables) mixed with dispersing or drying agents (*15*, *16*) or on cereals (*17*, *18*). However, PLE has never been applied for the determination of pesticides on seed coating.

Pesticides in extracts are then usually analyzed by gas chromatography (GC) or by high-performance liquid chromatography (HPLC). Single pesticide analytical methods have been widely studied. Fernandez-Alba (*6*) already analyzed imidacloprid residues in food by HPLC with diode-array detection (DAD) while Navalon (*19*) determined imidacloprid content in vegetables by GC−MS. As early as 1975, the development of the multiresidue method by Luke (*20*) has considerably reduced expenses and preparation times. The poor volatility, polarity and/or thermal stability of some compounds have made HPLC the most convenient method to analyze pesticides while the apparition of short analytical column—particle size and column length respectively inferior to 2.5 μm and 75 mm—has widely contributed to the reduction of analysis times (*21*).

Detection by diode-array or more generally by UV is very suitable for the multiresidue method (*6*, *8*, *9*). However, due to the

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low sensitivity of the UV detectors (*22*) and the presence of UV interferents in extracts, LC−MS techniques have been widely applied for the determination of a wide range of residual pesticides (*15*, *23*−*28*). Anyway, as the number of pesticides in coated seeds is generally between one and three and their content is very high, the use of UV detection is sufficient for the quantification of pesticides on seed coatings.

This study reports the development of simple, fast, efficient and inexpensive methods to extract and quantify pesticides on sampled loaded seeds by HPLC−UV. The aims of this work were as follows: (i) to evaluate the benefits of using a short chromatographic column for pesticide analysis by comparing its performance with this of conventional column; (ii) to study the influence of different parameters, like solvent for ultrasonic extraction and oven temperature for pressurized liquid extraction, in order to determine the optimum conditions for both extraction procedures; (iii) to evaluate the applicability of both methods for the quality control of loaded seeds.

MATERIALS AND METHODS

Chemicals. Pestanal grade standards of bitertanol, fludioxonil, imidacloprid, metalaxyl-M and tefluthrin were purchased from Fluka (Seelze, Germany). Purity was superior to 96.8% for all of them.

Solvents. Acetonitrile (synthesis grade for extraction and HPLC grade for analyses), ethyl acetate (synthesis grade for extraction) and acetone (synthesis grade for extraction) were purchased from Carlo Erba Reagents-SDS (Val de Reuil, France). Deionized water (≤18 MΩ·cm resistivity) was obtained from Millipore Simplicity water system (Molsheim, France).

Materials. Uncontaminated seeds of soft wheat (Apache variety) and corn (Masaba variety) were supplied by Epi de Gascogne (Francescas, France).

Representative seed samples (50 g) were milled with an Ika Werke M20 (Staufen, Germany).

Studied seed loading solutions containing one or two pesticides (written in parentheses) were supplied from the corresponding manufacturers: Gaucho Blé (imidacloprid and bitertanol) from Bayer Cropscience (Lyon, France), Celest (fludioxonil), Austral Plus (fludioxonil and tefluthrin) and Influx XL (metalaxyl-M and fludioxonil) from Syngenta (Saint-Cyr-L'Ecole, France). Since anthraquinone is present in some of the formulations, Pestanal grade anthraquinone (purity: 99.8%) was purchased from Fluka (Seelze, Germany) to be included in a mixed standard solution for the development of analytical methods.

Preparation of Stock and Working Standard Solutions. Individual stock standard solutions (ca. 2000 μg/mL) were prepared by weighing accurately 400 mg of each compound and dissolving them in 200 mL of synthesis-grade acetonitrile.

For wheat fortification, a mixed working standard solution was made in a 500 mL volumetric flask by diluting imidacloprid (125 mL), fludioxonil, bitertanol and tefluthrin (50 mL each) stock solutions and adjusting to volume with acetonitrile. The concentration of imidacloprid in working solution is higher because the level of this compound in the seed loadings is much higher.

For corn fortification, a working solution (500 mL) was prepared from metalaxyl-M and fludioxonil stock solutions (50 mL each) and adjusted to volume with acetonitrile.

The previous working solutions were used for the development of calibration curves and for the fortification of seeds. Calibration curves were plotted from standards in acetonitrile or in matrix extracts at six different levels in triplicate.

For the development of HPLC methods, a mixed standard solution was prepared by dissolving 2 mg of anthraquinone with 5 mL of each working solution and agitating for 5 min. All standard solutions were stored in the dark at +4 °C.

LC−UV. HPLC−UV analyses were all performed with a SCM1000 vacuum membrane degasser, a quaternary P4000 pump, an AS3000 autosampler and an UV2000 detector (Spectra Physics Analytical Inc., Fremont, CA). Data acquisition and processing were carried out with a computer using the Chromeleon version 6.70 software (Dionex, Sunnyvale, CA). Detection was carried out at a wavelength of 211 nm for all the components. This wavelength was selected to be convenient for all the proposed components. At this wavelength and for the studied concentration levels—between 10 and 700 mg/kg—the interference is not significative.

Separations with the short analytical column (33 × 4.6 mm i.d., 1.5 μm particle size) were performed on a Turbo 80 ODS3 (Cluzeau Info Labo, Sainte-Foy-la-Grande, France). The precolumn (25 × 4.6 mm i.d.) was packed with 0.5 μm stainless steel frits (0.094 in. disk diameter, 0.062 in. disk thickness, 0.250 in. o.d., Upchurch Scientific, Oak Harbour, WA).

An aliquot of 5 μL was injected into the HPLC system and eluted at 30 °C, with a flow rate of 1.3 mL·min^{-1} under the following gradient conditions, where A is ultrapure water and B is acetonitrile: t=0 min, A−B (90:10, v/v); t=0.5 min, A−B (60:40, v/v); t=4.5 min, A−B (50:50, v/v); t= 5 min, A−B (0:100, v/v); t = 7 min, A−B (0:100, v/v); t = 7.5 min, A−B (90:10, v/v); t = 9 min, A−B (90:10, v/v).

Separations with the traditional analytical column (250 × 4.6 mm i.d., 5 μm particle size) were performed on a Luna C18(2) (Phenomenex, Torrance, CA) protected by a Security guard cartridge C18 (4 mm × 3.0 mm i.d.) from Phenomenex (Torrance, CA).

An aliquot of 20 μL was injected into the HPLC system and eluted at 30 °C, with a flow rate of 1.3 mL/min under the following gradient conditions, where A is ultrapure water and B is acetonitrile: t=0 min, A−B (60:40, v/v); t=0.5 min, A−B (50:50, v/v); t=12 min, A−B (45:55, v/v); t= 13 min, A−B (0:100, v/v); t=17 min, A−B (0:100, v/v); t=17.5 min, A−B (60:40, v/v); t = 29 min, A−B (60:40, v/v).

Accelerated Solvent Extraction Procedure. Five grams of milled seeds was dispersed with 35 g of Sand of Fontainebleau (Prolabo, Fontenay-sous-Bois, France). The mixture was introduced in a 34 mL extraction cell and placed in the ASE 200E system connected to a four-bottle solvent controller (Dionex, Sunnyvale, CA). Cells were preheated for 5 min, and samples were extracted by cycling twice for 5 min at 120 °C and 1500 psi with acetonitrile. Nitrogen at a pressure of 10 bar was used to assist the pneumatic system, flush the cell (60% of the volume) and purge the system (100 s). The final extract (nearly 45 mL) was collected in a 60 mL glass vial with Teflon coated rubber caps.

Ultrasound-Assisted Extraction Procedure. For the extraction by ultrasonication, an aliquot of milled seeds (5 g) was introduced in a 50 mL erlenmeyer. Forty milliliters of acetonitrile was injected into the erlenmeyer, and the mixture was placed in an ultrasound bath (30 min) at room temperature and then vigorously stirred (1 h). The system was allowed to stand for 10 min, and the supernatant was removed. The solid phase was taken up twice with acetonitrile (2 × 5 mL). All the supernatants were collected to form the ultrasonication extract.

Sample Preparation. The extract (either PLE extract or ultrasonication extract) was introduced into a 50 mL Falcon tube and then evaporated in an EZ-2 Plus evaporator (Genevac Limited, Ipswich, U. K.) at 50 °C to dryness. Dry extract was reconstituted in 10 mL of acetonitrile, homogenized by ultrasonic dissolution and vortex agitation, filtered through an Acrodisc 0.45 μm filter (Pall Corporation, Ann Arbor, MI) to remove solid particles and analyzed by HPLC with no further purification.

Recovery Test. A sample of milled seeds was spiked with adequate working solution at two fortification levels (30 and 300 mg/kg, except for imidacloprid, 75 and 750 mg/kg) as follows: 5 g of uncontaminated seeds was spiked with 0.75 or 7.5 mL of the wheat or corn contaminating working solution. The system was gently stirred and then allowed to stand overnight to dryness. Five experiments were carried out individually at respective spiking levels.

Preparation of Loaded Seeds. Five hundred grams of seeds was coated *in situ* with seed loading solutions as follows: the coating solution was vigorously shaken and the necessary volume to treat 500 g of seed, according to the rates of use recommended by authorities (*29*−*32*), was incorporated in a flask. The volume of seed loading solution was adjusted to 7.6 mL with ultrapure water, and the mixture was agitated magnetically for 5 min and spread over the seeds. The mixture was manually shaken for 5 min to obtain a homogeneous coating on the seed. Seeds were allowed to stand overnight to dryness.

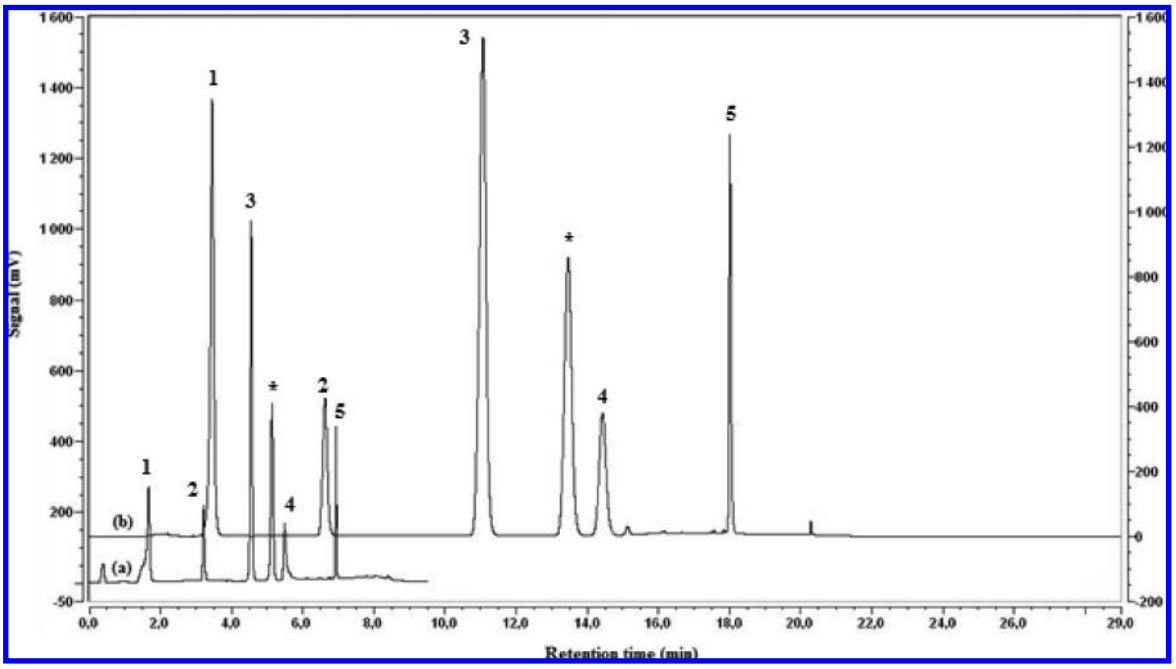

Figure 1. HPLC−UV chromatogram registered at 211 nm for a pesticide mix standard solution in acetonitrile with (**a**) short column Turbo ODS 80 chromatographic conditions and (**b**) Luna C18 (2) chromatographic conditions. Peaks: *, anthraquinone; 1, imidacloprid; 2, metalaxyl-M; 3, fludioxonil; 4, bitertanol; and 5, tefluthrin (chromatographic conditions as described in Materials and Methods).

RESULTS AND DISCUSSION

HPLC Performance. The chromatographic separation was fulfilled with a CIL Cluzeau Turbo ODS 80 column. The interest of this column is its low particle size (1.5 μm vs 3 or 5 μm for commonly used columns) so that the exchange surface is much higher and the column length is so scaled down (33 mm vs 150 or 250 mm) but the LC pressure remains acceptable (inferior to 150 bar). Performance of CIL Cluzeau short column was compared with a conventional column (Phenomenex Luna C18 (2), 250 × 4.6 mm i.d., 5 μm) at the same oven temperature (30 °C) and the same flow rate (1.3 mL/min). Chromatograms of a pesticide mixed standard solution in acetonitrile obtained with both HPLC systems are presented in **Figure 1**. Because of the presence of anthraquinone, a bird repellent, in some of the studied formulations, this compound was added to the mix solution for separation, but it was not quantified. Thanks to the short column, the time and the consumption of acetonitrile per analysis were both divided by 3 (i.e., 9 min vs 29 min for analysis time and 6.14 mL vs 20.78 mL for acetonitrile consumption). Considering a column lifetime of 1000 analyses, it represents a large benefit of more than 13.8 days and 14.6 L of acetonitrile. The peak resolutions ranged between 2.3 and 22.0 for the short column and ranged from 2.6 to 15.9 for the conventional column (data not shown). For both HPLC methods, all the resolutions were very similar, except for the tefluthrin peak resolution much higher with the short column system. Since the short column appeared to perform much better to quickly determine pesticides in extracts, this column was selected for our application.

Selection of Extracting Solvent. Recoveries and precisions for the extraction of pesticides by sonication with three different solvents (acetonitrile, ethyl acetate and acetone) were compared (**Figure 2**). Recoveries and RSDs were obtained by replicating extractions 5 times at each spiking levels. The lowest average recovery (73%) was obtained for the imidacloprid (at 75 mg/kg with acetone), whereas the highest recovery (102%) was for fludioxonil (at low fortification level with acetonitrile) with RSDs between 1% (fludioxonil with acetone) and 6% (metalaxyl-M with acetone). Acetone extraction yielded the lowest recoveries, ranging from 73% to 94%, whatever the pesticide and whatever the matrix. An increase in the extraction efficiency was generally observed from ethyl acetate (between 82% and 100%) to acetonitrile (from 85% to 102%) for every pesticide. In wheat, imidacloprid always gave lower recoveries than three other compounds with every solvent, maybe due to its polarity. In corn, metalaxyl-M was always slightly less recovered than fludioxonil. All the RSDs were between 1 and 6%, showing a good repeatability for the three extraction procedures. Considering the previous results of recovery tests and repeatability, although ethyl acetate extraction ensured very good results for every pesticide, recovery rates with acetonitrile were slightly better. So acetonitrile was considered as the optimum solvent for the extraction of pesticides on wheat and corn.

Evaluation of ASE Oven Temperature. Since acetonitrile was evaluated as the best solvent for the extraction of pesticides, it was selected to evaluate the influence of temperature on PLE extraction efficiency at 2 different fortification levels. **Figure 3** shows a certain influence of oven temperature on recovery for all the components. Indeed, the higher the temperature, the higher the pesticides were generally recovered. The recoveries at 40 °C ranged from 64% (imidacloprid in wheat at 75 mg/kg) to 86% (fludioxonil in corn at 300 mg/kg), whereas pesticides were recovered at 120 °C between 84% (imidacloprid in wheat at 75 mg/kg) and 102% (tefluthrin in wheat at 300 mg/kg). Recoveries were particularly influenced by oven temperature from 40 to 80 °C, with recoveries ranging from 82% (imidacloprid in wheat at 75 mg/kg) to 97% (fludioxonil on corn at 30 mg/kg). **Figure 3** shows recoveries were slightly increased from 80 to 120 °C. Similarly to ultrasonic extraction, recoveries for imidacloprid in wheat were slightly lower than for other compounds, whereas, in corn, recoveries for metalaxyl-M and fludioxonil were similar. No thermal degradation was noticed for any compound between 40 and 120 °C. Like Blasco et al. (*15*), an increase in color and in cloudy suspension was observed due to the coextraction of compounds of high molecular mass. RSDs all ranged from 1% to

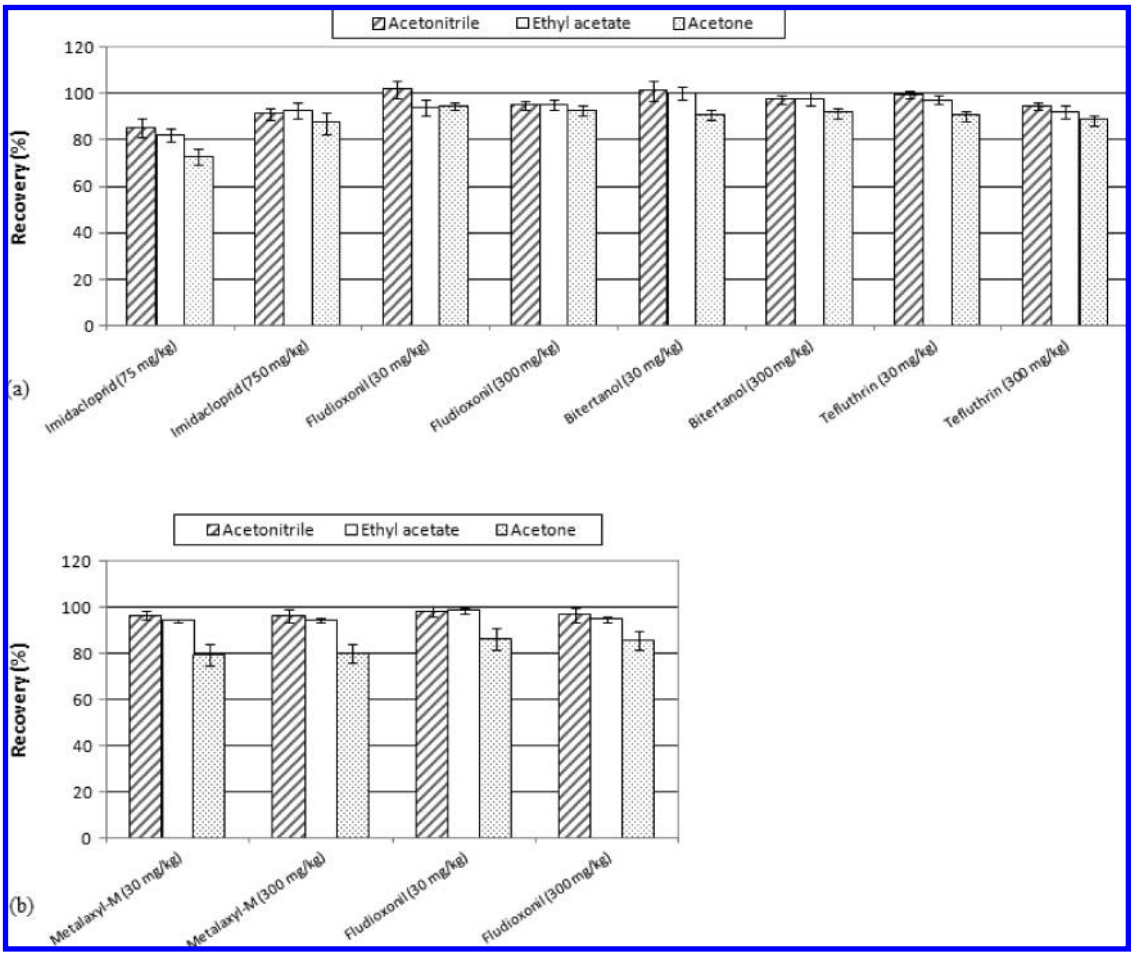

Figure 2. Effect of different solvents on the recovery of selected pesticides on (**a**) wheat and (**b**) corn at two different spiking levels.

9%, showing a good repeatability for the pressurized liquid extraction between 40 and 120 °C. Thus, the optimum PLE procedure was to extract preheated (5 min) pesticides with acetonitrile (60% flush) at 120 °C and 1500 psi for 5 min in two cycles. The different results obtained by both proposed pressurized liquid extraction (PLE) and ultrasonic extraction were absolutely similar in terms of efficiency (recoveries) and precision (RSDs).

Method Validation. Linearity of the proposed methods was assessed by the plotting of a matrix-matched external calibration curve. The curve was obtained from matrix extracts spiked at 6 different levels in triplicate. The linear dynamic ranges were between 0.3 and 1000 mg/kg for imidacloprid and between 0.12 and 400 mg/kg for all other pesticides. For ultrasonic extraction, the determination coefficients (r^2) were greater than 0.9984 for pesticides on wheat and greater than 0.9985 for pesticides on corn. Concerning the pressurized liquid extraction, determination coefficients were respectively higher than 0.9980 and 0.9987 for pesticides on wheat and on corn. These results show a good linearity for both selected extraction procedures. The limit of detection (LOD) was evaluated as the analyte concentration that is necessary to have a signal greater than three times the standard deviation of the noise level whereas the limit of quantification (LOQ) corresponds to a signal greater than ten times the standard deviation of the noise level (*33*). For ultrasonic extraction, limits of detection and quantification respectively ranged from 0.08 and 0.30 mg/kg (for imidacloprid in wheat) to 0.50 and 1.50 mg/kg (for tefluthrin in wheat and fludioxonil in corn), whereas for PLE, they ranged from 0.10 and 0.40 mg/kg (for fludioxonil in wheat) to 0.30 and 1.50 mg/kg (for tefluthrin in wheat). LOQs were always within the linear dynamic ranges so both proposed methods can be used in the selected calibration ranges.

Application of Proposed Methods on Loaded Seed Samples. Recoveries of pesticides on loaded seeds were the ratio of extracted pesticides out of the pesticides initially added with the seed loading solution. The effectiveness of both proposed extraction methods (ultrasonication with acetonitrile and PLE extraction with acetonitrile at 120 °C) was evaluated with different samples of loaded seeds. For the extraction by ultrasonication (**Table 1**), recoveries for coated pesticides on wheat ranged between 76% (tefluthrin on Austral Plus coating) and 95% (bitertanol on Gaucho Blé coating) with RSDs from 2% to 5%, whereas recoveries on corn ranged between 16% and 78% with 3% RSDs. Extraction recoveries for Gaucho Blé loaded pesticides were very similar to those of spiked uncontaminated seeds, while pesticides on Syngenta coatings (Celest, Austral Plus and Influx XL) were less extracted than spiking pesticides. The extraction for metalaxyl-M particularly shrank from 96% (recovery tests) to 16% (loaded pesticides). The poor extraction rate of pesticides for the 3 Syngenta coatings may be due to the particular polymeric sticking additives present in the formulations. These additives are known to improve the adhesion of pesticides on seeds and could limit, in our case, the extraction of pesticides on loadings. Consequently, the ultrasonic extraction of pesticides on loaded seeds appeared as a limited method in some cases.

Concerning the pressurized liquid extraction (**Table 2**), extraction of pesticides on loadings all ranged from 82% (fludioxonil on Influx XL coated corn seeds) to 95% (tefluthrin on Austral Plus coated wheat seeds) with RSDs between 2% and 7%. Recoveries

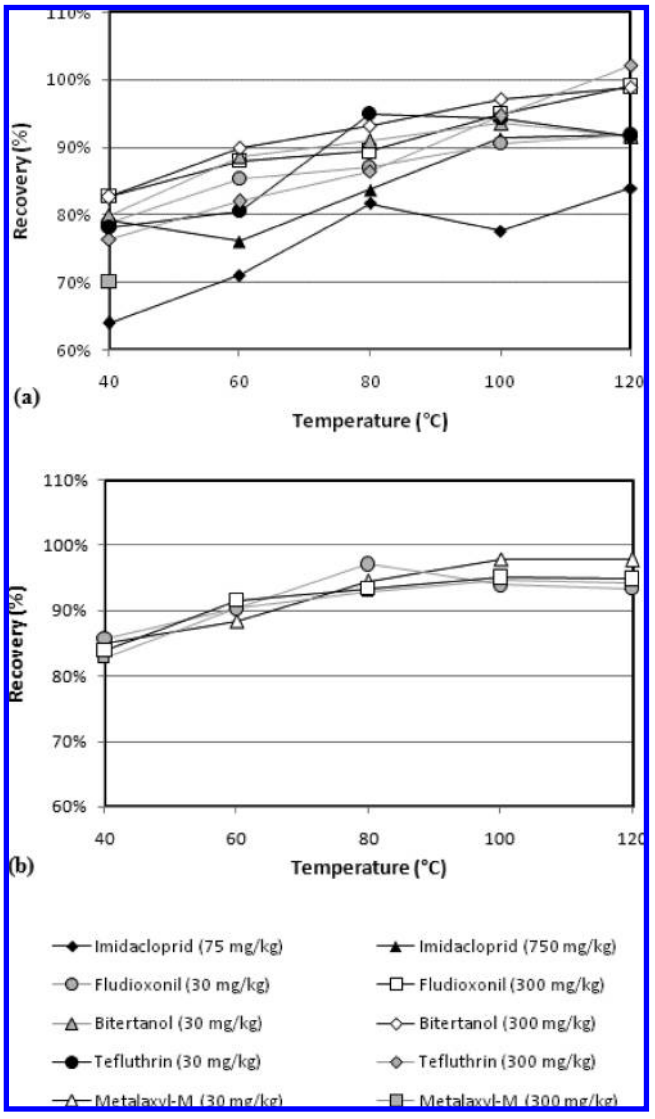

Figure 3. Effect of temperature on PLE efficiency on (**a**) wheat; (**b**) corn. Extraction conditions: acetonitrile (60% flush), 2 cycles, 5 min preheat time, 5 min static time.

Table 1. Recoveries and Repeatabilities (RSDs) of Pesticides Extraction on Coated Seeds for Ultrasonication Extraction (n = 5)

	wheat						corn	
	Gaucho Blé		Celest		Austral Plus		Influx XL	
pesticides	recovery (%)	RSD (%)	recovery (%)	RSD (%)	recovery (%)	RSD (%)	recovery (%)	RSD (%)
imidacloprid	87	5						
metalaxyl-M							16	3
fludioxonil			78	3	84	4	78	3
bitertanol	95	4						
tefluthrin					76	5		

of pesticides on seed loadings were a little lower than those of spiked uncontaminated seeds. However, recoveries of loaded pesticides by PLE were generally much higher than those obtained by ultrasonic extraction, except imidacloprid and bitertanol on Gaucho Blé loading. Consequently, a high oven temperature for PLE made the polymeric additives reach their glass transition temperature and ensured a better pesticide extraction.

In conclusion, the determination of pesticides in loaded soft wheat and corn seeds has been assessed by the HPLC system

Table 2. Recoveries and Repeatabilities (RSDs) of Pesticide Extraction on Coated Seeds for Pressurized Liquid Extraction (n = 5)

	wheat						corn	
	Gaucho Blé		Celest		Austral Plus		Influx XL	
pesticides	recovery (%)	RSD (%)	recovery (%)	RSD (%)	recovery (%)	RSD (%)	recovery (%)	RSD (%)
imidacloprid	85	5						
metalaxyl-M							84	3
fludioxonil			84	5	87	7	82	5
bitertanol	91	5						
tefluthrin					95	7		

equipped with the short column. Indeed, the comparison of both systems' performances exhibited a considerable reduction of analysis time and eluent consumption by using a short column without altering peak resolutions. Extracts were obtained by ultrasonication or by PLE with no further purification than filtration. Recovery tests for ultrasonic extraction exhibited an influence of solvent whereas recovery rates for pressurized liquid extraction showed an importance of oven temperature. Indeed, the best recovery rates were obtained for ultrasonic extraction with acetonitrile while the higher rates were observed for pressurized liquid extraction at high temperature (120 °C). Recoveries for ultrasonic extraction and pressurized liquid extraction were similar. Thus, the purchase of a PLE system is not necessary for the extraction of standard matched crops. However, in the case of loaded seeds, the extraction rates for ultrasonication were generally very low for some of the loaded pesticides, due to the presence of sticking agents in the loading formulation. Pressurized liquid extraction, on the contrary, presented results as good as recovery tests. Consequently, PLE appears as a valuable system for the extraction of a wide range of loaded pesticides.

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