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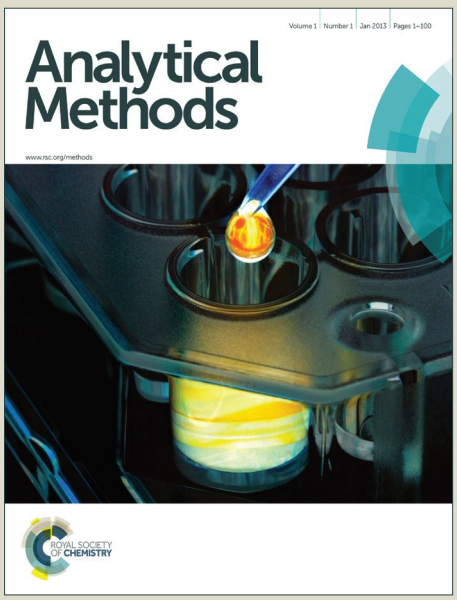

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Development of an on-line molecularly imprinted solid phase extraction by liquid chromatography-mass spectrometry for triazine analysis in corn samples

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ABSTRACT

A highly selective method for the analysis of triazine herbicides in corn samples based on molecularly imprinted solid phase extraction (MISPE) has been developed. Molecularly imprinted polymers (MIPs) were synthetized by precipitation polymerization using atrazine as a template, methacrylic acid as a functional monomer, ethylene glycol dimethacrylate as a crosslinker, and 2,2'-azobis-isobutrynitrile as an initiator. MISPE was developed for the on-line and automated enrichment of atrazine, simazine, terbutryn, simetryn and ametryn from corn sample extracts. High-performance liquid chromatography and time-of-flight mass spectrometry were used for the separation and confident determination of the herbicides. The limits of detection and quantitation of the proposed method were set to 1.6–3.3 μg kg^{-1} and 5.0-10.0 μg kg^{-1}. The method was successfully applied for the analysis of five types of corn and the recoveries of the triazines from the spiked samples ranged from 80.2 to 119.1%.

Keywords: Triazines, Corn, Molecularly imprinted polymers, On-line solid phase extraction, Liquid chromatography coupled to mass spectrometry.

ABBREVIATIONS USED

AIBN, 2.2'– azobisisobutyronitrile; **BAμE**, bar adsorptive microextraction; **DLLME**, dispersive liquid-liquid microextraction; **EGDMA**, ethylene glycol dimethacrylate; **EPA**, Environmental Protection Agency; **HAc**, Acetic acid; **HPLC**, high performance liquid chromatography; **LOD**, limit of detection; **LOQ**, limit of quantification; **LPME**, liquid-phase microextraction; **MAA**, methacrylic acid; **MEPS**, microextraction by packed sorbent; **MIP**, Molecularly imprinted polymer; **MISPE**, molecularly imprinted solid phase extraction; **NIP**, non-imprinted polymer; **RSD**, relative standard derivation;

SBSE, stir bar sorptive extraction; **SPE**, solid phase extraction; **SPME**, solid-phase microextraction.

1 INTRODUCTION

Triazine herbicides have been applied to the pre- and post-emergence control of weed for agricultural and non-agricultural purposes[1,2]. The intensive use of herbicides in large agricultural areas has raised concerns about their effects on the environment, as triazines and their degradation products are very toxic and stable for many years[3]. The Environmental Protection Agency (EPA) requires the tolerance of triazine herbicides until 0.25 mg kg^{-1} and the Brazilian Health Surveillance Agency (ANVISA) dictates the concentration of atrazine and simazine must not exceed 0.25 and 0.02 mg kg^{-1}, respectively, in corn crops[4]. Therefore, the development of simple, rapid and sensitive analytical methods for the determination of triazine herbicides[5–12].

Sample preparation is an important step in most analytical processes. The sample is treated prior to its analysis for the removal of interferences from matrix and to improvements in the selectivity of the analytical method[13]. According to the principles of green chemistry, the miniaturization of sample preparation techniques has been highlighted due to its low consumption of sample, solvents and reagents. A variety of methods, such as solid phase extraction (SPE)[14,15] solid-phase microextraction (SPME)[16], microextraction by packed sorbent (MEPS)[17], stir bar sorptive extraction (SBSE)[18], bar adsorptive microextraction (BAμE)[19], liquid-phase microextraction (LPME)[20] and dispersive liquid-liquid microextraction (DLLME)[21] can be applied for the preconcentration and clean-up of the analytes in different samples. Allied to the miniaturization advantages, the automation of analyses has showed to be a reliable

approach for the avoidance of a multistep and time-consuming sample preparation. In this context, on-line SPE is an attractive alternative and a trend to the current analytical methods.

The use of on-line SPE has enabled the development of faster methods and increases the sample throughput. Therefore, several papers reporting on-line SPE with applications in environmental and food analyses have been published[22–24]. The development of new sorbent materials aims at enhances selectivity, adsorption capacity, simplicity, robustness, resistance to a wide range of pH, temperatures and solvents, and physical-mechanical stability at low cost[25]. Several sorbents can be used in on-line SPE; however, in recent years, MIPs (molecularly imprinted polymers) have been demonstrated as a promising sorbent in on-line-SPE[26–32] and applications in chromatographic stationary phases[33], chiral separations[34], antibody mimics[35] and drug delivery systems[36].

The present manuscript addresses the synthesis of novel MIPs for the simultaneous determination of atrazine, ametryn, simazine, simetryn and terbutryn in corn samples. On-line MISPE followed by LC-ESI-TOF separation/detection were used in the procedure. The optimization of the effective parameters were investigated by chemometric tools. The column switching MISPE-LC-ESI-TOF method was validated according to ANVISA RE899 for the analysis of triazines in corn samples.

2 MATERIALS AND METHODS

2.1 Reagents and Standards

Pesticides simazine, simetryn, ametryn and terbutryn were purchased from Sigma-Aldrich (Steinheim, Germany) and their stock solutions (100 mg L^{-1} concentration) were

prepared in acetonitrile obtained from Tedia (Fairfield, OH, USA) and stored in the dark at 4°C. Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA) and 2.2' – azobisisobutyronitrile (AIBN) were obtained from Sigma-Aldrich (Steinheim, Germany). Acetic acid (HAc) was purchased from Merck (Darmstadt, Germany) and used for the preparation of the mobile phase. Ultrapure water purified by a Milli-Q plus system (Millipore Bedfort, MA, USA) was used in all experiments. Strata X from Phenomenex (Torrance, CA, USA), C18 from Alltech (Deerfield, IL, USA) and alumina from Merck (Darmstadt, Germany) were used as sorbents in some experiments.

2.2 Instrumentation

The pesticides were quantified by an LC-ESI-ToF system. A Shimadzu LC system (Kyoto, Japan) equipped with three LC-20AD pumps, an SIL-20AC autosampler, a CTO-20A oven, a CBM-20A system controller, and a six-port switching valve from Valco (Houston, TX, USA) was employed for the experiments. A micrOTOF-QII hybrid quadrupole/time-of-flight (QqToF) system fitted with an electrospray ionization (ESI) source, all from Bruker Daltonics (Bruker, Germany) provided the mass spectra data. Data Analysis 4.2 software, also from Bruker Daltonics, controlled all the events in the chromatographic system.

Synthesized MIPs were packed into a stainless steel column (20 mm × 4.6 mm, 2.0 µm frits) by a slurry packing technique. An LC-20AD delivery solvent (Shimadzu, Japan) and methanol as a packing solvent were used for the evaluation of their chromatographic characteristics.

Chromatographic separations were carried out with a C18 column (150 mm x 2.1 mm, 5 µm, from Nano Separation Technologies (NST), Sao Carlos, Brazil) and a mobile phase composed of an acetonitrile/water mixture (70:30, v/v) with 0.1% acetic

acid (HAc) at 0.2 mL min^{-1} flow rate. The temperature was set at 35°C and a 50 µL injection volume was used. The optimized MS conditions used for the method validation were positive ESI mode, 4.5 kV capillary voltage, 200 °C desolvation temperature, desolvation gas at 8 L h^{-1} and nebulizer gas at 4 bar.

2.3 Preparation of imprinted polymers

The current MIP synthesis was based on precipitation polymerization. The polymers were synthesized by mixing 0.5 mmol (0.1 g) of atrazine (template) and 2 mmol (0.18 g) of MAA (functional monomer), both dissolved in 30 mL of acetonitrile. The mixture was stored for 12 h at 4°C. Subsequently, 10 mmol (1.92 g) of EGDMA (cross-linker) and 0.12 mmol (20 mg) of 2-azoisobisbutonitrile (initiator) were added. The solution was degassed ultrasonically and purged with nitrogen for 10 min. The flask was sealed under nitrogen and the mixture was heated at 60°C for 24 h. The polymers were washed with methanol/acetic acid (90:10, v/v) and dried at 60°C. This procedure is schemed in Figure 1. An analogous procedure was employed for the synthesis of non-imprinted polymer (NIP), in the absence of the atrazine. The study of their selectivity coefficient, characterization and comparison with other sorbents in reported in[17].

2.4 Sample preparation and chromatographic parameters

The extraction method employed the solid corn matrix was previously described and simply based on solvent extraction[17]. The corn extract sample (50 µL) was directly introduced into an MISPE column by an HPLC autosampler at 0.2 mL min^{-1} using 0.1% acid acetic in water. Eluent A was used as carrying and washing solvent (0 – 5 minutes). The commutation valve was set to the load position and the matrix interference compounds were discarded. The valve was switched to the transference position (5 – 7

minutes) and the extracted triazines were eluted from the MISPE column to the analytical column in the backflush elution mode by the analytical mobile phase. The chromatographic separation was perfomed by a mixture of acetonitrile/water (70:30, v/v) with 0.1% acetic acid (eluent C) as the mobile phase at 0.2 mL min^{-1} flow rate. The valve was then switched to the load position (7 – 22 minutes). While the analytes were being separated and detected by the C18 column and ToF analyzer, the MISPE column was cleaned with acetonitrile (eluent B) for the removal of any residual interference and conditioned with 0.1% acid acetic in water (eluent A) for the next sample extraction. Both columns were kept in an oven at 35°C during all analysis. Figure 2 shows the system's configuration.

2.5 Optimization of the MISPE-HPLC procedure

The optimization step for the quantitative MISPE extraction was perfomed by a full two-level fractional design (2^3) and involved the followings variables: length of column (20, to 80 mm), loading pump flow rate (0.15 at 0.30 mL min^{-1}) and extraction time of the solid matrix (6 to 12 h). The length of the column (20, 40 to 60 mm) and the extraction time (2, 4, 6, 8 and 10h) showed significant effects and were further optimized by a Doehlert matrix. The experimental set of data was processed by Statistica 8.0 software (StatSoft, Tulsa, USA).

3 RESULTS AND DISCUSSION

3.1 Optimization of the MIP-SPE procedure

The full fractional design 2^3 estimated three factors, namely length of column, loading pump flow rate and extraction time matrix. Ten experimental levels were performed

with a duplicate at the central point. The effects of the variables on the screening experiments are shown in the Supporting Information (Figure S1) in the form of a Pareto chart representing the behavior of each variable evaluated for all analytes. The results show a negative effect of the length of the column on all pesticides, which indicates an increase in the pre-column caused band broadening and a decreased in the peak intensity. Therefore, shorter columns must be used to improve the efficiency of the chromatographic system, so that the sensitivity due to adequate sorption of the solid extraction phase is not compromised. Simetryn and terbutryn also showed a negative effect on the extraction time, i.e. a longer extraction time for the removal of the pesticides from corn samples is not necessary. All variables were optimized by a Doehlert matrix. As the effect of the loading pump flow rate was not significant, it was fixed at 0.30 mL min^{-1} for all experiments.

The analysis of variance (ANOVA) revealed that the quadratic model was adequate. The response surface obtained by the Doehlert matrix is shown in the Supporting Information (Figure S2, which represents the model for atrazine). The optimum values for the evaluated factors were 40.0 mm column length and 6 h of extraction time with organic solvent.

3.2 Method validation

The MISPE-HPLC-UV method for the analysis of triazine corn samples was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), intra- and interday precision and accuracy, recovery and matrix effects[37], according to ANVISA.

The calibration curves were constructed using data obtained from spiked samples in six different concentrations (n=5) each level and 10.0 – 500.0 µg kg^{-1} range. All coefficients of determination (R^2) were ≥ 0.9913. The limit of quantification (LOQ, S/N

= 10) and limit of detection (LOD, S/N = 3) were 5.0-10.0 μg kg^{-1} and 1.6-3.3 μg kg^{-1}, respectively. Table 1 summarizes the detection limit of some methods for the determination of triazines in different samples[6, 38–41].

The intra- and inter-day accuracy (n=5) and precision (n=5) were determined through the analysis of triazines in three different concentrations - low, medium and high (10, 120, 500 μg kg^{-1}) - in two consecutive days. The results, expressed as the percentage of the relative standard derivation (RSD) for precision and as bias (% bias). Low variability (RSD <14.6%) and adequate accuracy (-1.3 to -9.5) were obtained. Such values are under the requirements of the FDA guidelines for precision and accuracy, i.e., < 20% at LOQ level and <15% higher concentration. Experiments were performed for the evaluation of the efficiency relative of the process and the results are shown in Table 2.

Five corn samples were obtained from different supermarkets, however, as shown in Figure 3a, traces of pesticides could not found in real corn samples. It is noteworthy the method developed showed levels below those permitted by the legislation of LMR established by ANVISA. Therefore, the real samples were fortify at two different concentrations for the evaluation of the method. Figure 3b shows the total ion chromatograms from triazine herbicides at 20 μg kg^{-1} obtained by MISPE-LC-ESI-ToF. Blank corn samples were spiked with five triazines at different fortified concentrations (10 and 500 μg L^{-1}) for the development of recovery studies. The results of atrazine, ametryn, simazine, simetryn and terbutryn ranged from 80.2 to 110.9%, 86.0 to 119.1%, 80.5 to 111.5%, 87.4 to 113.0%, and 87.2 to 107.5%, respectively, Table 3.

4 CONCLUSIONS

The application of a new molecularly imprinted material for the selective on-line solid-phase extraction (MISPE) of triazine herbicides in corn samples has been demonstrated. The developed, optimized and validated method could successfully detect triazines at low concentration levels in corn samples in agreement with all figures of merit evaluated. It is a promising method that employs MIPs for the selective sorption and further confident determination of triazines by an on-line MISPE-LC-ToF set up using an automated column-switching approach.

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FIGURE CAPTIONS

Figure 1. Scheme of MIP synthesis.

Figure 2. Configuration of the chromatographic system for column-switching MISPE (Molecularly Imprinted Solid Phase Extraction) in backflush mode.

Figure 3. (a) Ion extracted chromatogram of real corn samples (b) Ion extracted chromatogram spiked sample at 20 μg kg^{-1} from on-line MISPE-LC-ESI-TOF. Peak identification (m/z): simazine (202.085±0.005), simetryn (214.109 ±0.005), atrazine (216.099 ±0.005), ametryn (228.128 ±0.005) and terbutryn (242.145±0.005). Chromatographic conditions: NST C18 column; 0.2 mL min^{-1} flow rate: acetonitrile/water (70:30 v/v) mobile phase and 50μL injection volume.

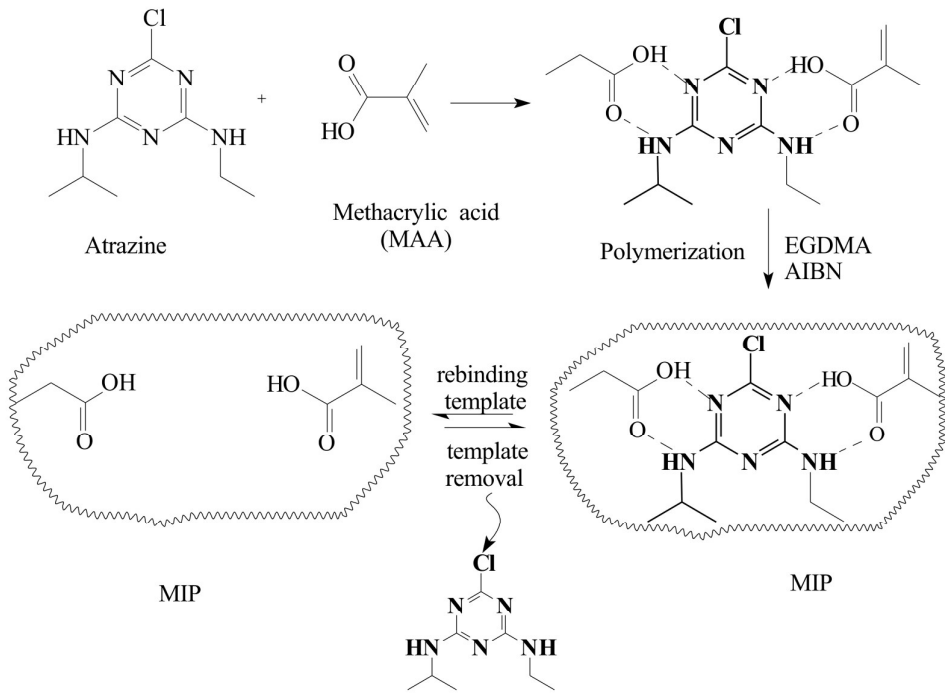

Figure 1

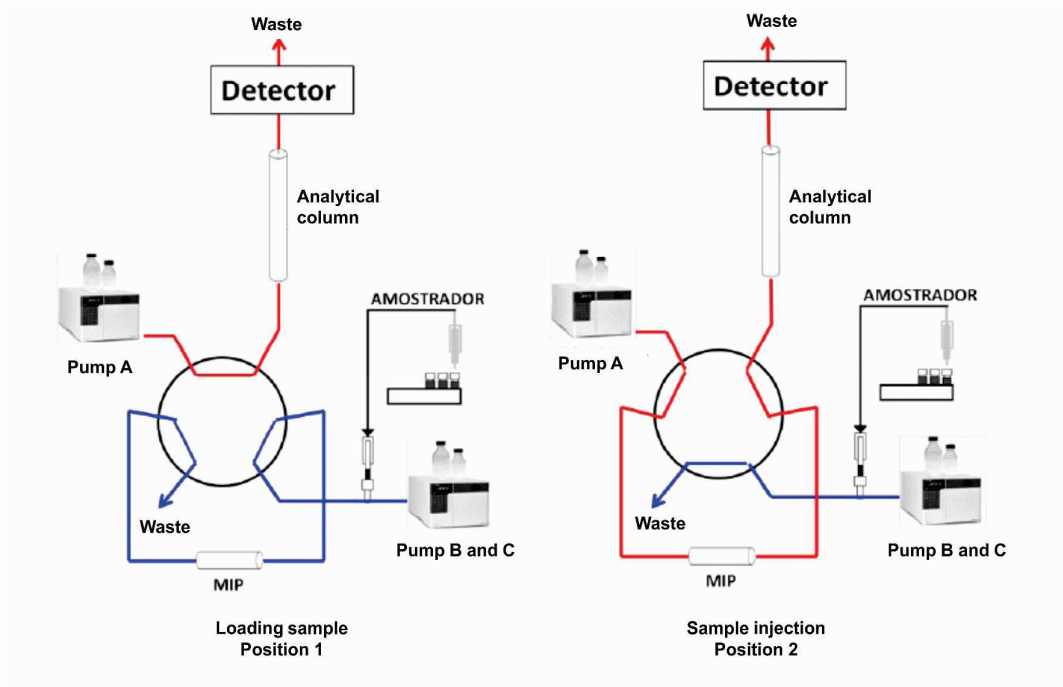

Figure 2

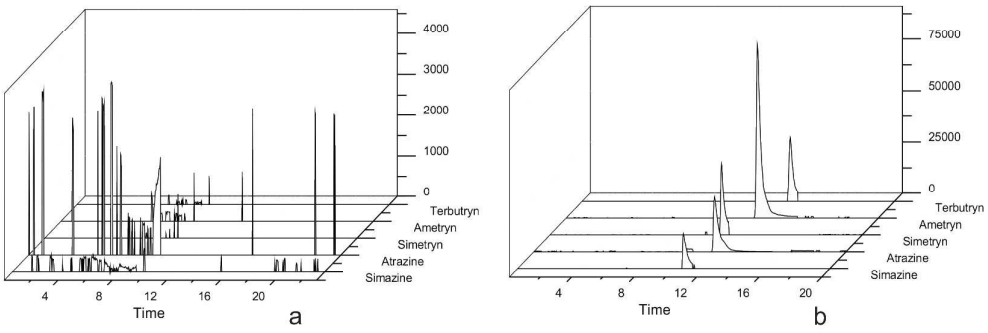

Figure 3

Table 1 - Comparison of methods for analyses of triazines in different matrix.

Phases	Extraction type/Method	Sample	LOD	LOQ	Ref.
MIP	UPLC-MS/MS	Herbal plants	0.003 mg kg^{-1}	-	6
MIP	SPE-HPLC	Sugar cane juice	5.0-50.0 µg L^{-1}	20-150 µg L^{-1}	38
-	DLLME-HPLC	Honey	5.3-8.4 µg kg^{-1}	-	40
-	MAE-HPLC	Soil	0.16-0.3 µg mL^{-1}	0.5-1.0 µg mL^{-1}	41
MIP	MIM-HPLC	Corn	5.8 µg kg^{-1}	-	42
MIP	*on-line* MISPE-LC-TOF	Corn	1.6-3.3 µg kg^{-1}	5.0- 10.0 µg kg^{-1}	This work

Table 2 - Validation figures of merit: precision, accuracy, and relative efficiency of the process (n=5)

		1 st day		2 sta day		Average (n=10)		
Triazines	**Level ($\mu g\ kg^{-1}$)**	**Accuracy intra day B[a] (%)**	**Precision intra day RSD, %**	**Accuracy intra day (%) B[a] (%)**	**Precision intra day (%) RDS, %**	**Accuracy inter day B[a] (%)**	**Precision inter day (%) RSD, %**	**Relative efficiency of the process (%)**
Atrazine	10.0	-5.0	1.4	-5.2	1.6	-7.1	1.5	-
	120.0	-1.9	10.6	-2.1	13.3	-9.5	11.9	98.4
	500.0	-3.7	11.6	-2.4	14.1	-8.9	12.8	-
Ametryn	10.0	-5.2	12.4	-6.5	2.9	-9.5	7.7	-
	120.0	-4.8	11.6	-3.0	11.4	-7.4	11.5	100.7
	500.0	-2.7	2.8	-3.2	9.9	-2.7	10.9	-
Simazine	10.0	-4.5	7.6	-6.9	2.2	-7.3	4.9	-
	120.0	-3.5	11.3	-3.4	7.0	-3.4	11.7	91.5
	500.0	-3.2	14.1	-3.9	10.5	-3.6	12.3	-
Simetryn	10.0	-6.5	10.5	-2.6	10.4	-8.7	10.4	-
	120.0	-2.6	12.4	-1.8	12.2	-9.3	12.3	96.9
	500.0	-4.0	12.6	-2.9	14.6	-3.6	13.6	-
Terbutryn	10.0	-7.1	0.4	-6.4	2.8	-8.0	1.6	-
	120.0	-1.3	8.9	-1.8	12.4	-7.6	9.7	97.9
	500.0	-2.0	10.0	-2.6	14.3	-4.1	9.2	-

B[a]: *bias* of the method, RSD: relative standard deviation

Table 3 - Recoveries of triazines obtained by the analysis of spiked corn samples (n=5).

		Corn 1	Corn 2	Corn 3	Corn 4	Corn 5
Triazines	**Spiked ($\mu g\ kg^{-1}$)**	**B[a] % (RSD, %)**	**B[a] % (RSD, %)**	**B[a] % (RSD, %)**	**B[a] % (RSD, %)**	**B[a] % (RSD, %)**
Atrazine	10	-15.3 (3.6)	-19.8 (6.3)	+10.9 (8.1)	+3.8 (7.5)	8.2 (9.1)
	500	-7.6 (5.2)	-13.8 (3.7)	-8.8 (2.4)	-7.4 (5.1)	-1.1 (1.3)
Ametryn	10	+6.2 (1.4)	+12.2 (5.3)	+14.0 (3.3)	+17.6 (4.1)	+19.1 (5.3)
	500	-11.3 (4.1)	+1.2 (2.0)	-14.5 (3.6)	-1.4 (1.8)	-6.2 (1.9)
Simazine	10	-19.5 (7.4)	-16.3 (8.4)	-17.6 (5.8)	-5.5 (1.5)	-9.3 (7.4)
	500	+11.5 (4.9)	+9.8 (1.8)	+10.5 (3.4)	+8.4 (1.2)	5.1(3.9)
Simetryn	10	+9.6 (4.9)	+7.5 (9.9)	+13.0 (13.5)	+6.1 (6.1)	+7.4 (2.1)
	500	-8.7 (5.1)	-11.4 (0.5)	-1.7 (6.0)	-7.3 (5.1)	-12.6 (4.3)
Terbutryn	10	+7.5 (6.5)	-2.5(7.3)	-1.1 (7.0)	-3.2 (2.8)	-5.5 (8.4)
	500	+4.2 (5.3)	-7.3 (4.3)	-5.9 (1.5)	-12.8 (3.1)	-8.1 (1.8)

[a] B: *bias* of the method, RDS: relative standard deviation

7x2mm (300 x 300 DPI)