Analytical Methods

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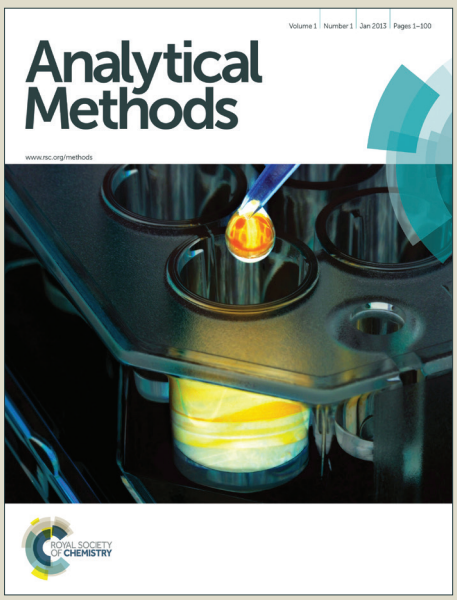

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Title:

"Trace analysis of pesticides and an assessment of their occurrence in surface and drinking waters from the State of São Paulo (Brazil)"

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Abstract

An efficient method based on solid phase extraction (SPE) and determination by liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been developed for simultaneous determination of 12 pesticides at trace levels in surface and drinking waters from the State of São Paulo (Brazil), which are likely to be contaminated due to the widespread use of these products. Several parameters that affect SPE and the analysis were studied, such as conditioning and elution solvents, sample pH, breakthrough volume and matrix effects. Method development was validated by several figures of merit. Recoveries from synthetic samples spiked at 150 ng L^{-1} and 1,000 ng L^{-1} levels with difenoconazole, epoxiconazole, tebuconazole, atrazine, azoxystrobin, pyraclostrobin, picoxystrobin, trifloxystrobin, profenofos and fipronil varied from 73 to 99 %, with intraday precision in the 5 - 24 % range. A lower fortification level (10 ng L^{-1}), close to detection limits, led to recoveries from 86 - 155 %, which was considered acceptable for the purpose of trace analysis of environmental samples. Low detections limits (1 - 50 ng L^{-1}) and quantification limits (2 - 180 ng L^{-1}) were obtained. The method was applied for the determination of pesticide residues at the nanogram per liter level in samples of drinking water from 9 cities and in surface waters from 13 rivers of the State of São Paulo, Brazil. The results showed that the investigated waters are highly impacted with carbendazim and atrazine, which were the most frequently determined compounds.

Keywords: pesticides, drinking water, surface water, SPE, LC-MS/MS, trace analysis

1. Introduction

The use of pesticides is of fundamental importance to sustain modern agricultural practices, including those of Brazil, to maintain high productivity. Currently, different chemical substances are used to control a specific set of pests; these substances can reach surface waters and cause adverse effects to non-target organisms, such as aquatic biota. Furthermore, the chronic exposure to some pesticides may interfere in the endocrine systems of humans and animals at nanogram per liter levels.

Brazil is the largest consumer of pesticides in the world and 465 active ingredients are currently approved by the Ministry of Agriculture for use on different crops [1]. However, Brazilian water quality guidelines do not contemplate numerous products used routinely, resulting in a lack of standards for pesticides with high probabilities of occurrence in surface and drinking waters [2, 3]. The selection of contaminants that should be regulated is not an easy task. It is necessary to consider the amount of substance used, its potential hazard to non-target species, its physical-chemical characteristics and its occurrence in the aquatic environment. Although Brazil leads the world in pesticide consumption, little is known about the presence of pesticides in Brazilian water bodies and their potential to be removed by drinking water treatment plants. To assess water quality considering the presence of contaminants at trace levels, reliable and sensitive analytical methods are required and a validation process is an important step when an official method that comprises numerous analytes does not exist. Recent studies have reported analytical methods for the determination of organic contaminants at the ng L^{-1} level using liquid chromatography-tandem mass spectrometry (LC-MS/MS), a technique which provides suitable selectivity for the determination of these contaminants in complex matrices [4-11]. Sample preparation using solid phase extraction (SPE), combined with LC-MS/MS determination, has enabled the development of multi-residue methods for the determination of trace amounts of dozens of pesticides simultaneously in different aquatic matrices [5, 6, 12-16]. Nowadays, different extraction cartridges have been used to obtain high recovery rates in the sample preparation step [12-16]. Sonication [12], solid-phase microextraction

[17] and QuEChERS [18] are other methods of sample preparation that have been used in environmental samples for the determination of pesticides. However, the limits of detection reported were higher than those obtained when using SPE and LC-MS/MS. Moreover, there is a lack of official methods for the determination of non-regulated contaminants.

The objective of this work was to develop and validate an analytical method for the determination of 12 pesticides at the nanogram per liter level using solid phase extraction and liquid chromatography coupled to tandem mass spectrometry (SPE-LC-MS/MS), by optimizing the most suitable conditions for their determination in real samples, like river and drinking waters, which were analyzed and then used to obtain data about the occurrence of these pesticides in different samples collected in the State of São Paulo, Brazil. The selected compounds were atrazine, carbendazim, chlorpyrifos, profenofos, difenoconazole, epoxiconazole, tebuconazole, azoxystrobin, picoxystrobin, pyraclostrobin, trifloxystrobin and fipronil, which are approved for use in Brazilian crops. The list includes some of the most consumed ones, whose sales in the State of São Paulo varied between 35 and 1676 tons in 2012 [19].

2. Experimental

2.1. Reagents and chemicals

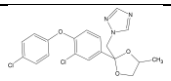
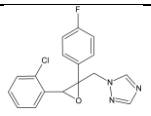
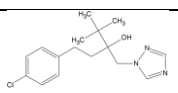
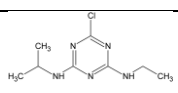
High purity standards of 12 pesticides: atrazine (98.8 %), carbendazim (97.0 %), chlorpyrifos (99.2 %), profenofos (96.9 %), difenoconazole (97.0 %), epoxiconazole (99.0 %), tebuconazole (99.7 %), azoxystrobin (99.7 %), picoxystrobin (99.9 %), pyraclostrobin (99.9 %), trifloxystrobin (99.5 %) and fipronil (97.9 %) were purchased from Sigma–Aldrich (Steinheim, Germany). Chromatographic grade methanol (MeOH) and acetonitrile were supplied by Merck (Darmstadt, Germany). Acetone (99.9 %) was purchased from Tedia (Fairfield, USA), formic acid (98 %) from Sigma Aldrich (Steinheim, Germany) and ammonium formate (98 %) from Riedel-de Haën (Germany).

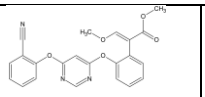
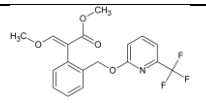
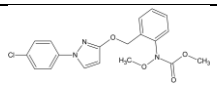
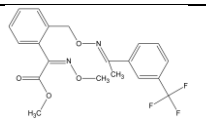
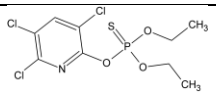
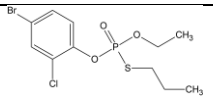
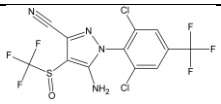
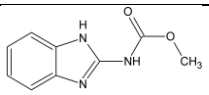
Individual stock solutions (400 mg L^{-1}) of each pesticide were prepared from the appropriate solid standard in methanol and stored in amber glass bottles at −4 °C. A mixture containing 10 mg L^{-1} of each of the 12 compounds was prepared daily as the working solution in methanol by dilution of the individual stock solutions and was used to spike samples and to prepare analytical curves. Calibration solutions (500, 100, 50, 10, 5 and 1 µg L^{-1}) were prepared by adding variable volumes of mixed working solutions to 70/30 (v/v) H_2O/MeOH solution, which represents the initial mobile phase composition used for chromatographic analysis.

2.2. Selection of pesticides

For validation of a multi-residue method, representative pesticides were selected based on a list of the most consumed pesticides of São Paulo State, Brazil [19, 20]. The selected pesticides belong to different types including acaricides, insecticides, fungicides and herbicides from six different chemical groups: triazoles (difenoconazole, epoxiconazole and tebuconazole), triazines (atrazine), strobilurins (azoxystrobin, picoxystrobin, pyraclostrobin and trifloxystrobin), organophosphates (chlorpyrifos, profenofos), phenyl pyrazoles (fipronil) and benzimidazoles (carbendazim). Relevant physical chemical properties for the selected pesticides are presented in Table 1.

Table 1: Physico-chemical properties of the selected pesticides [21]

Pesticides	Chemical group	Molecular Formula	Chemical Structure	CAS number	Vapor Pressure (mPa)	Henry's law constant (Pa m^3/mol)	Log K_{ow}	Water solubility (mg L^{-1})	pKa
Difenoconazole	Fungicide	$C_{19}H_{17}Cl_2N_3O_3$		119446-68-3	$3.3x10^{-5}$	$9.0x10^{-7}$	4.36	15	1.0
Epoxiconazole	Fungicide	$C_{17}H_{13}ClFN_3O$		133855-98-8	$1.0x10^{-2}$	$4.7x10^{-4}$	3.30	7.1	NA
Tebuconazole	Fungicide	$C_{16}H_{22}ClN_3O$		107534-96-3	$1.7x10^{-3}$	$1.0x10^{-5}$	3.70	36	NA
Atrazine	Herbicide	$C_8H_{14}ClN_5$		1912-24-9	$3.9x10^{-2}$	$1.5x10^{-4}$	2.70	35	1.7

Azoxystrobin	Fungicide	$C_{22}H_{17}N_3O_5$		131860-33-8	$1.1x10^{-7}$	$7.3x10^{-9}$	2.50	6.0	NA
Picoxystrobin	Fungicide	$C_{18}H_{16}F_3NO_4$		117428-22-5	$5.5x10^{-3}$	$6.0x10^{-4}$	3.60	3.1	$3.98x10^{3}$
Pyraclostrobin	Fungicide	$C_{19}H_{18}ClN_3O_4$		175013-18-0	$2.6x10^{-5}$	$5.3x10^{-6}$	3.99	1.9	NA
Trifloxystrobin	Fungicide	$C_{20}H_{19}F_3N_2O_4$		141517-21-7	$3.4x10^{-3}$	$2.3x10^{-3}$	4.50	0.6	NA
Chlorpyrifos	Insecticide	$C_9H_{11}Cl_3NO_3PS$		2921-88-2	$2.7x10^{0}$	$6.7x10^{-1}$	1.82	1.4	NA
Profenofos	Insecticide, Acaricide	$C_{11}H_{15}BrClO_3PS$		41198-08-7	$2.5x10^{0}$	$1.7x10^{-3}$	1.70	28	NA
Fipronil	Insecticide, Veterinary treatment	$C_{12}H_4Cl_2F_6N_4OS$		120068-37-3	$2.0x10^{-3}$	$2.3x10^{-4}$	3.75	3.8	NA
Carbendazim	Fungicide	$C_9H_9N_3O_2$		10605-21-7	$9.0x10^{-2}$	$3.6x10^{-3}$	1.48	8.0	4.2

NA: Not Applicable

2.3. Sampling and sample preparation

Samples were collected in amber glass bottles (1 L), previously washed with ultrapure water, ethanol (99 %) and acetone, and heated at 400 ^{o}C for 4 hours. Samples were transported in a cooler and kept under refrigeration until extraction, which was performed within 24 h.

River and drinking water samples were collected every fourth month in the period of January to December (2013), including periods of dry and wet. Surface water samples were collected in 13 rivers in the São Paulo State: Atibaia River, Capivari River, Corumbataí River, Piracicaba River, Jaguari River, Camanducaia River, Mogi Guaçu River, Mogi Mirim River, Cachoeira Creek, Tabajara Creek, Pires Creek, Pinhal Creek and Tatu Dam. Near to these rivers there is significant agricultural activity, predominantly sugar cane, coffee, soya and citrus crops. Annual physical-chemical parameters (pH, dissolved oxygen and turbidity) from the studied rivers are available from

the São Paulo State Environmental Agency reports [22]. Figure 1 shows the sampling sites in the map of the State of São Paulo, Brazil.

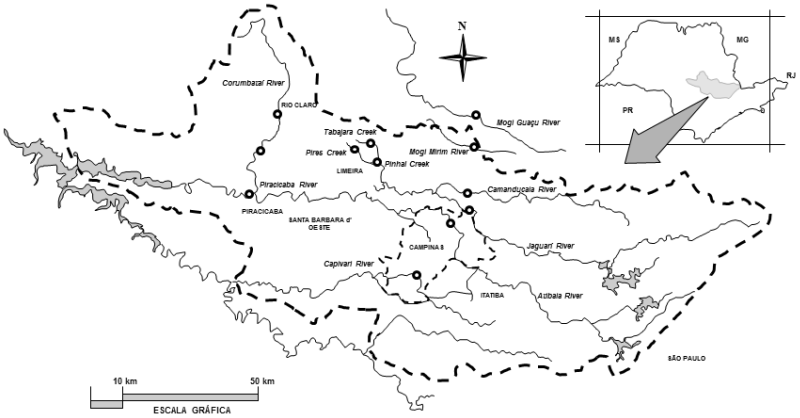


Figure 1: Localization of the sampling sites in the State of São Paulo, Brazil

Drinking water samples were collected from 9 cities in the State of São Paulo, *i.e.*, Campinas, Espírito Santo do Pinhal, Itatiba, Ribeirão Preto, São Paulo, Limeira, Santa Barbara D'Oeste, Rio Claro and Piracicaba.

One liter of each sample was filtered using glass fiber filters (Sartorius Stedim Biotech, Goettingen, Germany) and extracted by solid phase extraction prior to chromatographic analysis.

2.4. Solid-phase extraction (SPE)

To establish the best SPE conditions, parameters such as type of solid phase, conditioning and elution solvents, breakthrough volume and initial pH of the samples were studied.

2.4.1. Cartridges and solvents

Four cartridges types containing 500 mg of extraction phase were studied: Oasis HLB (Waters, Milford, USA), Strata SAX (Phenomenex, Torrance, USA), C18 Envi-18 (Supelco, Bellefonte, USA) and Envi Carb (Supelco, Bellefonte, USA). Two solvents were studied: methanol and

acetonitrile. Artificial mixtures (synthetic samples) were prepared in ultra pure water containing 10 µg L^{-1} of each of the 12 selected pesticides. In the first test, the four different cartridges were conditioned with 6.25 mL of each solvent individually (methanol or acetonitrile), then 125 mL of the synthetic sample were passed through the solid phase at 10 mL min^{-1}. All cartridges were dried for 20 min under a gentle stream of ultra-pure nitrogen gas (99.998 %). Pesticides were eluted with a 6.25 mL aliquot of the same solvent used in the conditioning step. The elution step was carried out using a 12-port Prep Sep vacuum manifold (Fisher Scientific, Fair Lawn, USA) with appropriate pre-cleaned glass tubes. Solvents were carefully evaporated to dryness with a gentle flow of ultra-pure nitrogen gas and the recovered target compounds were re-suspended to a final volume of 5 mL of the 70/30 (v/v) H_2O/MeOH solution.

According to the results obtained in the preliminary experiments for the SPE development step, the further experiments with synthetic samples and the real samples analysis were performed by using 500 mg/6 mL Oasis HLB cartridges, conditioned with both solvents, *i.e.*, 5 mL of methanol followed by 5 mL of acetonitrile and 5 mL of ultrapure water. Synthetic samples were percolated through the solid phase at 10 mL/min, the cartridge was dried for 20 min under a stream of ultra-pure nitrogen and eluted with 4 mL of methanol followed by 4 mL of acetonitrile. After that, the solvents were evaporated until dryness with a gentle flow of ultra-pure nitrogen gas and the recovered target compounds were re-suspended to a final volume of 0.4 mL with the 70/30 (v/v) H_2O/MeOH solution.

2.4.2. pH of the samples

The pH of the samples varied from 5.5 to 6.0. Acidification to ~ pH 3 was included as an analytical parameter to verify the best conditions of SPE extraction, expressed as percentage of recovery. The experiments were performed using 500 mL of the synthetic samples prepared in ultrapure water containing 300 ng L^{-1} of each of the selected compounds. Two groups were studied:

in one group the SPE extraction was carried without adjustment of the pH, and in another group, synthetic samples had the pH adjusted to about 3 by the addition of formic acid.

2.4.3. Breakthrough volume

One important parameter in SPE is the breakthrough volume. When analyzing environmental matrices, high volumes are necessary for representative sampling and sufficient detectability [23]. To evaluate if a 1 L volume would cause possible losses of the selected pesticides, increasing ultrapure water sample volumes were enriched with the same mass of each compound [24]. Thus, an aliquot of 150 µL from a 1000 µg L^{-1} stock solution was added to 50, 100, 250, 500, 750 and 1000 mL of ultrapure water, separately. The results were evaluated comparing the recovery as a function of the sample volume.

2.5. LC-MS/MS determination

The LC–MS/MS analysis was performed using an Agilent 1200 Series LC system coupled to an Agilent 6410 triple quadrupole mass spectrometer with an electrospray ionization source (ESI). The software MassHunter was used to control the instrument and to evaluate the chromatographic and mass data. The chromatographic separation was performed in a thermostated column compartment (TCC G1316A) at 30 °C, using a reversed phase Zorbax SB-C18 column (2.1×30 mm, particle size of 3.5 µm) from Agilent Technologies and carried out with gradient elution using water and methanol. Three mobile phase additives (0.01 % formic acid, 0.1 % formic acid and a buffer solution composed of formic acid (0.01 %):ammonium formate (5 mmol L^{-1}) were evaluated and the performance was indicated by the sensitivity of the analytical curve. The solvents used as mobile phase were filtered through 0.2 µm nylon membranes (Sigma Aldrich, Steinheim, Germany). Stepwise gradient elution at a flow rate of 0.3 mL min^{-1} was programmed by increasing the relative organic solvent concentration from 30 % to 60 % in 1.2 min, maintaining for 3 min, followed by an

increase to 70 % in 3.5 min, and held constant for another 4 min. After re-adjusting to the initial conditions, the system was re-equilibrated for 5 min. The injection volume was 10 µL.

After the chromatographic separation, the pesticides were ionized using an electrospray ionization source (ESI) operating in the positive ion mode for all compounds except for fipronil, which was ionized in the negative mode. The following parameters were adjusted to maximize ionization: drying gas flow rate of 10 L min^{-1}, drying gas temperature of 350 °C, nebulizing gas pressure at 20 psi, and capillary voltage of 4000 V. Nitrogen was used as collision gas. Multiple reaction monitoring (MRM) transitions were employed for confirmation and quantification of the target compounds.

2.6. Validation study

The method performance was evaluated using the following validation parameters: analytical curve, linearity, limit of detection (LOD), limit of quantification (LOQ), matrix effect, precision (repeatability and intermediate precision) and accuracy (recovery). The analytical curves were obtained in triplicate at ten concentration levels between 0.5 and 250 µg L^{-1}. Satisfactory linearity was assumed if the linear correlation coefficient (r) value was higher than 0.99. Method accuracy and precision were evaluated using synthetic samples (ultrapure water) spiked at low, medium and high concentration levels (10, 150 and 1000 ng L^{-1}, respectively) with three replicates for 150 and 1000 ng L^{-1} and five replicates for 10 ng L^{-1}. Acceptance criteria for accuracy and precision were concentration level dependent [25]. For medium and high fortifications, methods were considered accurate if recovery was 70 - 130 % and precise if RSD < 20 %. As the intended use of the method is the assessment of pesticide occurrence in waters and the determination of these contaminants in the environment, which are expect to occur close to detection limits, the acceptable range for the lowest level (10 ng L^{-1} fortification) was 50 - 150 % for recovery and, according to the Horwitz equation [26], precision acceptability was RSD < 60 %.

Blank samples using ultrapure water were previously analyzed and no significant peaks at the selected transitions were observed. The LOQ of the method was determined considering 10 times the intercept of the regression line divided by the slope of the analytical curve prepared using standard solutions with 70/30 (v/v) H_2O/MeOH as solvent [27].

2.6.1. Matrix effect study

The matrix effect was investigated by comparing standards in solvent, 70/30 (v/v) H_2O/MeOH, to matrix-matched standards using the relative responses (matrix response / solvent response). As the matrix blanks analyzed contained some of the selected pesticides, experiments employing standard additions were carried out to evaluate the extent of the matrix effect and the dilution factor needed to minimize it. To obtain a representative matrix-matched standard, 8 L of surface water from the Atibaia River were collected using a continuous and constant sampling mode [28] during 18 h. Then, separately, 8 extractions of 1 L each were done using an Oasis HLB cartridge. After elution, each final eluate was combined in a single flask. The volume was then reduced until dryness and re-suspended in 2.4 mL of 30/70 (v/v) H_2O/MeOH corresponding to 300 µL for each of the eight extractions, resulting in a combined eluate. To obtain the matrix matched solutions in concentrations of 1, 5, 10, 50 and 100 µg L^{-1} of added compounds, 100 µL of the selected solution were added to 300 µL of the combined eluate. A blank of the sample was prepared by mixing 100 µL of 30/70 (v/v) H_2O/MeOH and 300 µL of the combined eluate. This represents a concentration factor of 2,500. The remaining combined eluate was 10 fold diluted and the same concentrations of added standard were prepared, representing a 250 fold concentration factor. The same process was repeated for drinking water produced with the same surface water.

The matrix effect was analyzed by comparing analytical curve sensitivity between external standard (α_s) and standard addition (α_M).

$$\text{Matrix effect}(\%) = \left(\frac{\alpha_M}{\alpha_s} - 1 \right) x100$$

3. Results and discussion

3.1. LC-MS/MS optimization

The separation performance was evaluated in terms of mobile phase eluotropic strength. A binary phase containing: (i) water with an additive to improve the ionization of the target compounds and (ii) an organic solvent that changes the polarity of mobile phase during the gradient program. Methanol was used as organic solvent and three additives were evaluated: 0.01 % formic acid, 0.1 % formic acid and a buffer solution composed of formic acid (0.01 %):ammonium formate (5 mmol L^{-1}). The sensitivity of the analytical curves was used to select the most suitable additive.

The use of 0.01 % formic acid as additive provided higher sensitivity for triazoles (tebuconazole, epoxiconazole, difenoconazole) and triazines (atrazine). For the determination of carbendazim, 0.01 % formic acid or the buffer solution gave the same sensitivity. For strobirulins, the buffer solution provided higher sensitivity but 0.01 % formic acid also provided acceptable levels of sensitivity, thus 0.01 % formic acid was chosen for the method. The buffer solution used as additive provided the best conditions for determination of chlorpyrifos and profenofos. The analytical curves are shown in the supplementary materials.

Confirmation and quantification of target compounds were carried out by mass spectrometry using the MRM mode and the instrumental parameters such as precursor ion, product ion and its respective collision voltage, for each transition, was optimized for the 12 selected pesticides (Table 2). The fragmentor parameter used for the determination of all compounds was 100 V. The chromatographic separation is shown in Figure 2 using the MRM profile obtained for the quantification transition from a 150 ng L^{-1} standard.

Table 2: Selected LC–MS/MS experimental parameters for each pesticide

Pesticides	Retention time (min)	Precursor ion (m/z)	ESI mode	Product ion (m/z)	Collision energy (eV)	Relative abundance (%)
Carbendazim	0.85	192.1	(+)	160.1 132.1	5 30	- 19.5

				105.1	35	11.1
Atrazine	5.34	216.2	(+)	174.1 103.9	15 15	- 13.5
Azoxystrobin	6.54	404.2	(+)	372.0 344.1	5 20	- 31.1
Epoxiconazole	7.98	330.1	(+)	121.2 101.2	20 35	- 45.7
Fipronil	8.46	435.0	(-)	250.0 330.0	25 25	- 62.6
Picoxystrobin	8.48	368.2	(+)	145.0 205.0	25 5	- 37.0
Tebuconazole	8.81	308.2	(+)	70.0 124.9	20 30	- 5.6
Pyraclostrobin	9.63	388.0	(+)	163.3 194.1	10 20	- 42.1
Difenoconazole	10.4	406.2	(+)	251.1 338.0	25 15	- 1.0
Trifloxystrobin	10.7	409.2	(+)	186.2 145.2 206.2	10 15 40	- 56.1 19.1
Profenofos	11.2	373.0	(+)	97.0 223.2 305.0	35 35 10	- 4.1 0.3
Chlorpyrifos	13.1	350.0	(+)	97.0 198.0 124.9	25 20 25	- 94.5 7.1

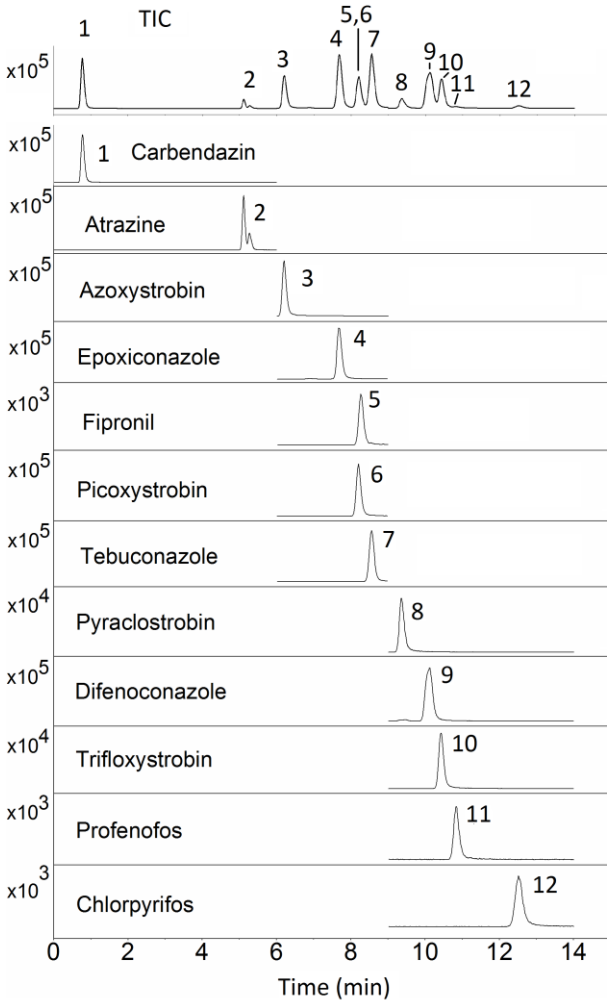


Figure 2: LC-MS/MS total ion chromatogram and MRM mode at the m/z of the quantification transition, of a 150 ng L^{-1} mixture of each pesticide

3.2. Optimization of sample preparation

Sample preparation is an important step to achieve high efficiencies and satisfactory recoveries in trace analysis determinations of organic compounds. In this study, sample preparation was optimized in terms of sorbent type in combination with two different elution solvents used in SPE extraction, sample pH adjustment prior to extraction and sample volumes. This study was performed using synthetic samples spiked with the 12 selected pesticides in ultra pure water.

3.2.1. Cartridges and solvents

In the SPE extraction step, four sorbents and two solvents were evaluated. Among the sorbents evaluated, the Envi-carb sorbent provided poor recoveries, up to 30 % (Figures 3a, 3b). For the other three sorbents (Oasis HLB, Strata SAX and Envi-18), no significant differences were observed (t-test, $p > 0.05$), except for atrazine and carbendazim that showed higher recoveries using Oasis HLB. Therefore Oasis HLB was chosen as an adequate sorbent for these pesticides. In terms of solvents for extraction, the use of acetonitrile provided the best results in terms of recovery for the majority of the selected pesticides while the use of methanol provided satisfactory recoveries for nine pesticides (Figure 3a, 3b). Thus, a second experiment evaluated the recovery of the selected compounds using Oasis HLB sorbent with methanol followed by acetonitrile as the elution solvent. This condition provided satisfactory recovery (> 70 %) for the all pesticides except for difenoconazole, chlorpyrifos and carbendazim (Figure 3c). However, Dujakovi'c *et al.* (2010) also obtained lower recoveries of carbendazim using methanol:acetonitrile in comparison with methanol only [14]. Chlorpyrifos was not evaluated in this study, but its recovery using methanol followed by acetonitrile was evaluated and it is shown in Figure 3c.

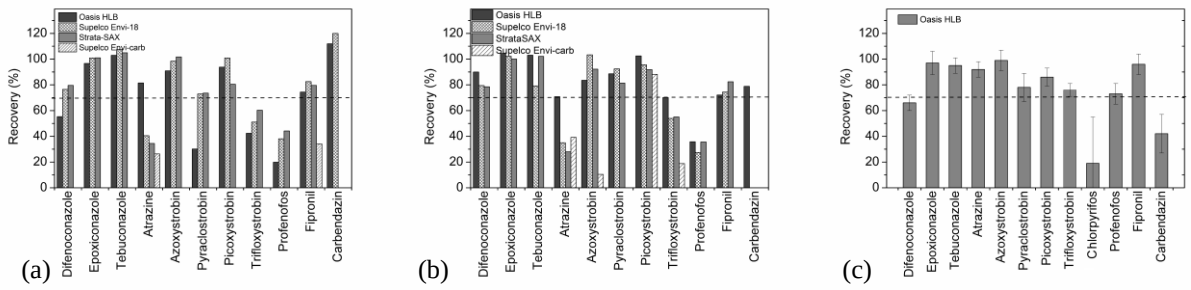


Figure 3: Recoveries (%) of the 12 selected pesticides using four extraction sorbents (Oasis HLB, Envi-18, Strata-SAX and Envi-carb) in association with two elution solvents (a) methanol and (b) acetonitrile. The recoveries using Oasis HLB sorbent and both methanol and acetonitrile are shown in (c). The dashed line indicates the minimum acceptable recovery percentage

3.2.2. Sample pH

Overall process efficiency [29] of the spiked samples of ultra pure water, drinking water and river water containing 300 ng L^{-1} of each of the 12 pesticides is presented in Table 3. No significant differences were observed when comparing both pH studied; hence samples without pH adjustment were applied for the method (t-test, $p > 0.05$).

Table 3: Overall process efficiency (%) for the 12 selected pesticides spiked in ultrapure water, river and drinking water samples

Pesticides	Overall process efficiency (%)					
	Ultrapure water		River water		Drinking water	
	without pH adjustment	pH 3	without pH adjustment	pH 3	without pH adjustment	pH 3
Difenoconazole	26	41	25	28	39	28
Epoxiconazole	61	87	54	55	58	53
Tebuconazole	64	67	60	57	61	52
Atrazine	72	90	53	45	65	53
Azoxistrobin	62	91	64	66	65	54
Pyraclostrobin	30	36	34	44	93	49
Picoxystrobin	46	50	45	53	65	58
Trifloxistrobin	25	23	25	33	65	48
Chlorpyrifos	3	4	4	11	9	1
Profenofos	32	41	32	45	48	48
Fipronil	93	99	50	72	96	106
Carbendazim	45	45	30	48	26	30

3.2.3. Breakthrough volume

In case breakthrough does not occur with the different volumes tested, the recoveries should remain constant for a given compound. Breakthrough did not occur for atrazine, fipronil and pesticides from the strobilurin and triazol classes. Profenofos and chlorpyrifos were the compounds with higher retentions, hence they presented the lowest recoveries. Figure 4c shows that carbendazim breakthrough occurs between 250 and 500 mL. This is the most polar analyte among the selected pesticides, hence the most likely to be carried by water during extraction.

The results showed that there was no significant loss of recovery caused by breakthrough for all pesticides, except for carbendazim (Figure 4), whose recovery decreased quickly with increasing

sample volume. Thus, a volume of 1 L was adopted for real samples in order to obtain higher concentration factors for the majority of compounds.

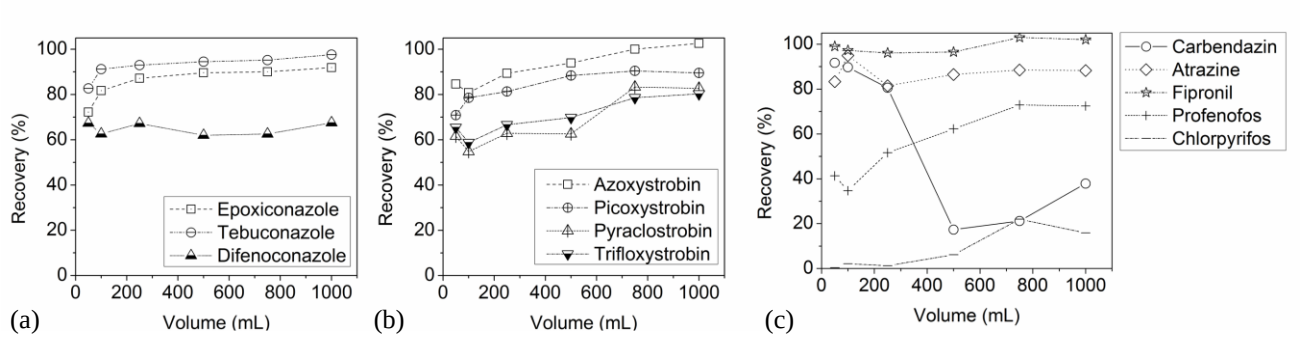


Figure 4: Recovery (%) of the 12 selected pesticides in relation to the sample volumes extracted by SPE: (a) triazoles; (b) strobilurins; (c) other studied groups

3.3. Analytical method performance

Method performance was evaluated considering equipment performance, validation studies and matrix effects, which are discussed in the following paragraphs.

3.3.1. Equipment performance and validation studies

As far as the instrumental parameters were concerned, the instrumental detection limit (IDL) and the instrumental quantification limit (IQL) were obtained using standard solutions, which varied between 2 and 445 pg for the column; intraday precision (% RSD) varied from 0.4 to 3, and interday precision (4 days, % RSD) varied from 4 to 23; linearity showed a correlation coefficient higher than 0.99 for all pesticides, except for profenofos, whose value was 0.978 (Table 4).

In terms of analytical method, recovery was tested for three concentrations levels: 10, 150 and 1,000 ng L^{-1}. At the lower level (10 ng L^{-1}), close to detection limits, recoveries from 37 % (carbendazim) to 156 % (epoxiconazole) were obtained, with RSD between 3 % and 66 %. For higher levels, recovery varied between 42 % and 99 %, with a RSD not higher than 24 %. Carbendazim and chlorpyrifos presented the poorest values of recovery; the first due to compound losses caused by breakthrough and the latter because it presented high retention in the cartridge. For

the establishment of limits of detection (LOD) and limits of quantification (LOQ), the matrix effect, which will be discussed at the next section (3.3.2), was taken into account. After the calculation described in section 2.6.1, the dilution factor of 10 fold was applied to obtain the final values described at Table 4.

3.3.2. Matrix effect

In LC-MS/MS, the matrix effect is usually caused by interference of the matrix components that coeluate with analytes and therefore compete with them during the ionization process. The number of the analyte ions can be reduced by interaction with matrix ions, causing ion suppression, or the signal can be increased by the presence of matrix ions, resulting in a negative or positive matrix effect, respectively [30, 31].

Due to the difficulty to find a matrix blank, matrix effects were calculated in terms of standard addition curve sensitivity instead of peak area or matrix-matched calibration. In this work, the matrix effects were evaluated by comparing solvent and standard addition sensitivities for the analytical curves and were expressed as the percentage by which the response of an analytes in pure solvent was altered due to the matrix components. If negative values were found, the matrix caused analyte signal suppression; if positive values were found, the matrix induced signal enhancement; if both responses agreed, no matrix effect occurred [32]. Matrix effects were evaluated for river and drinking waters, for 2,500 and 250-fold pre-concentration factors, respectively, aiming at a compromise between satisfactory detectability and minimum signal suppression.

A typical behavior of compounds that exhibited considerable matrix effects is exemplified with the difenoconazole results (Figure 5a). Analytical curves obtained using standards prepared in solvent (initial mobile phase composition) were used as reference of no signal suppression, hence presenting the highest sensitivity. When surface and drinking water were 2,500 fold pre-concentrated, interfering compounds caused difenoconazole signal suppression and thus a sensitivity

decrease. For this pre-concentration level, non-acceptable matrix effects were observed for surface water (-63 %) and drinking water (-73 %). However, when the matrix was 10 fold diluted, corresponding to a 250-fold sample pre-concentration, matrix effects decreased to -25 % and -9 % levels, respectively, and were considered satisfactory. A clean-up step on the SPE using a moderate solvent, such as water, could have been used to minimize the matrix effect, but it was not done in this work. Epoxiconazole, tebuconazole, atrazine, fipronil, profenofos and chlorpyrifos presented this behavior and matrix effect values are listed in Table 4. Carbendazim occurred in surface water at higher concentrations than the spike levels in the standard addition, hence matrix effects were not calculated for the 2,500 fold pre-concentration factor.

For the strobilurin class, typical behavior is shown in Figure 5b, exemplified by azoxystrobin. The parallelism of the curves showed a similar sensitivity, hence no significant matrix effects were observed for these compounds. Azoxystrobin occurred in both river and drinking waters, as can be seen from the standard addition curves, which are shifted at the y axis, compared to the solvent curve.

Another Brazilian study made by Silveira et al. showed high matrix effects for surface and drinking waters in the South region when pharmaceuticals were determined at trace levels [33].

Table 4: Figures of merit of the developed method. Instrumental detection limit (IDL), instrumental quantification limit (IQL), intraday and interday precision, linear correlation coefficient (r), recovery, limit of detection (LOD), limit of quantification (LOQ) and matrix effects for both river and drinking waters

Pesticides	Analytical Method										Matrix Effect (%)			
	Instrumental parameters					Recovery (%) (RSD)[a]			LOD [c] (ng L^{-1})	LOQ [c] (ng L^{-1})	River water		Drinking water	
	IDL (pg)	IQL (pg)	Intraday precision (RSD)	Interday precision (RSD)	Linear correlation coefficient (r)	10 ng L^{-1}	150 ng L^{-1}	1000 ng L^{-1}			2500x[b]	250x[c]	2500x[b]	250x [c]
Difenoconazole	1.6	5.4	2	5	0.999	102 (59)	66 (6)	72 (19)	1	2	-63	-9	-73	-25

Epoxiconazole	5.2	17	2	5	0.998	156 (55)	97 (9)	90 (14)	2	7	-38	-3	-43	-16
Tebuconazole	2.2	7.3	0.4	4	0.998	92 (3)	95 (6)	94 (14)	1	3	-56	-6	-66	-17
Atrazine	5.2	17	1	4	0.998	121 (51)	92 (6)	85 (12)	2	7	-74	-18	-67	-32
Azoxystrobin	6.7	22	3	6	0.997	152 (66)	99 (8)	97 (13)	3	9	-11	1	-10	-12
Pyraclostrobin	1.9	6.5	2	6	0.999	114 (62)	78 (11)	76 (19)	1	3	4	0.1	14	-14
Picoxystrobin	5.1	17	1	7	0.998	129 (51)	86 (7)	80 (17)	2	7	-27	-0.5	-6	-16
Trifloxystrobin	1.6	5.4	1	8	0.999	86 (35)	76 (5)	73 (24)	1	2	-24	-1	-34	-16
Chlorpyrifos	6.7	22	1	20	0.992	-	-	25 (23)	3	9	-56	-7	-71	-28
Profenofos	11	38	3	9	0.978	87 (25)	73 (8)	75 (18)	5	15	-43	-4	-46	-17
Fipronil	133	445	1	23	0.990	155 (39)	96 (8)	86 (16)	50	180	-86	-13	-52	-3
Carbendazim	1.6	5.2	1	5	0.999	37 (35)	42 (15)	69 (12)	1	2	-	-26	-71	-40

a: intraday precision

b: 2,500 fold pre concentration factor

c: 250 fold pre concentration factor

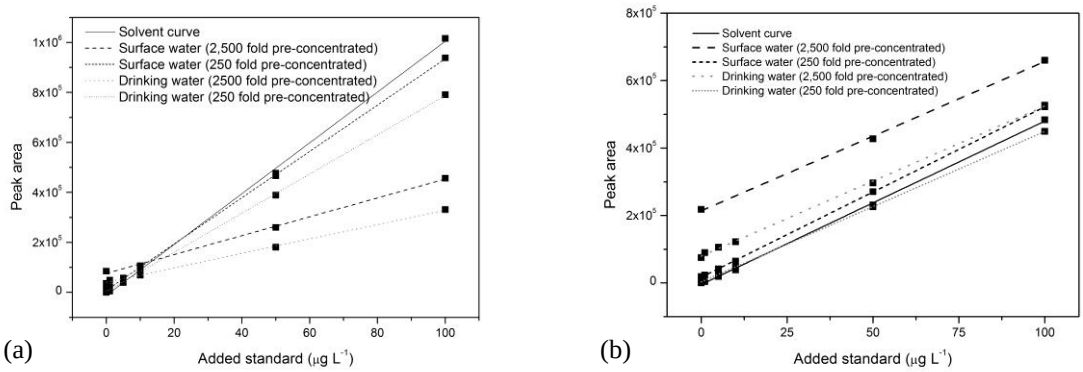


Figure 5: Analytical curves obtained in solvent as well as in two different matrices: surface and drinking water with 250 and 2,500 fold pre-concentration factor for (a) difenoconazole and (b) azoxystrobin

3.4. Application to real samples

The analytical method was successfully applied for drinking and river water analysis and the selected pesticides could be determined in nanogram per liter levels. The rivers investigated

presented nine of the twelve compounds analyzed and the concentrations varied from 3 to 293 ng L^{-1}. Chlorpyrifos, profenofos and fipronil were under their limits of quantification. For drinking water samples, three of the twelve pesticides (tebuconazole, atrazine and carbendazim) were determined in concentrations from 4 to 87 ng L^{-1} (Table 5).

Carbendazim was the most frequent contaminant detected as it occurred in 85 % of the river waters investigated and in 5.6 % of the drinking waters sampled. It can be noticed that carbendazim concentration levels in real samples can be underestimated (due to breakthrough and signal supression) and the values should only be considered as preliminary observations. The high frequency of detection of this compound shows a contamination scenario of concern for southern Brazilian rivers. Atrazine was the second most detected pesticide in river waters, with a frequency of detection of 46 %, and for drinking water, the frequency was 50 %. A study made by Caldas et al. investigated pesticides in surface waters from the South of Brazil and also detected carbendazim, atrazine, epoxiconazole and tebuconazole. These authors also detected epoxiconazole and tebuconazole in drinking water [34].

The only selected pesticide that is regulated in surface water in Brazil is atrazine, with a maximum allowed concentration of 2,000 ng L^{-1} [3]. For drinking water, five of our selected compounds are included in the regulation: carbendazim, atrazine, tebuconazole, profenofos and chlorpyrifos [2]. All the concentrations found for those five compounds in the drinking water samples analyzed were below the maximum allowed concentrations.

Table 5: Concentrations (ng L^{-1}) of the pesticides in river and drinking water samples from São Paulo State, Brazil

Pesticide	Rivers water samples (n=46)				Drinking water samples (n=18)			
	Frequency of detection (%)	Mean[a]	Minimum	Maximum	Frequency of detection (%)	Mean[a]	Minimum	Maximum
Difenoconazole	4.3	11	5	17	0		< LOQ	
Epoxiconazole	4.3	16	7	25	0		< LOQ	
Tebuconazole	13	8	3	19	16.7	8	4	16
Atrazine	46	47	7	293	50	26	7	87

Azoxystrobin	8.7	13	9	37	0		< LOQ	
Pyraclostrobin	2.2	5	5	5	0		< LOQ	
Picoxystrobin	6.5	3	<LOQ	7	0		< LOQ	
Trifloxystrobin	2.2	9	9	9	0		< LOQ	
Chlorpyrifos	0		< LOQ		0		< LOQ	
Profenofos	0		< LOQ		0		< LOQ	
Fipronil	0		< LOQ		0		< LOQ	
Carbendazim	85	82	3	781	5.6	9	9	9

[a] Values below LOQ excluded from calculation.

4. Conclusions

A rapid and efficient method based on solid phase extraction and liquid chromatography-tandem mass spectrometry was validated, allowing the determination of 12 pesticides with different physico-chemical properties in surface and drinking waters. The determination of residues of the selected pesticides by LC-MS/MS was satisfactory, allowing the confirmation and the quantification through the MRM acquisition mode, by monitoring at least two ion transitions for each compound studied. The greatest advantage of this method was the possibility of simultaneous determination of different pesticides classes (acaricides, insecticides, fungicides and herbicides) in nanogram per liter levels. Low detections limits (1 - 5 ng L^{-1}) and quantification limits (2 - 180 ng L^{-1}) were obtained with satisfactory recoveries and precision for ten compounds. The method was applied for 64 real samples collected in the state of Sao Paulo, and the results showed that the river waters investigated were mostly impacted with carbendazim and atrazine. For drinking waters, atrazine was the most detected analyte.

The method developed can be used in other studies involving to the determination of pesticides of Brazilian waters, thus enriching knowledge about the presence and fate of these contaminants in surface and drinking waters. Our findings also provide some occurrence information on not yet regulated pesticides and induce the establishment of water quality criteria for surface and drinking water in Brazil.

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