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High-performance liquid chromatographic determination of pesticides in tomatoes using laboratory-made NH_2 and C_{18} solid-phase extraction materials

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Abstract

A method for high-performance liquid chromatographic (HPLC) multiresidue determination of six pesticides in tomatoes was developed and validated. Silica-based C_{18} (octadecyl) and NH_2 (aminopropyl) solid-phase extraction (SPE) sorbents, made in our laboratory, were used for sample preparation. The SPE materials were obtained by thermal immobilization of appropriate polysiloxanes onto 40 μm silica surfaces and were used in sample preparation for multiresidue analysis of the following pesticides: tebuthiuron and diuron (urea herbicides), simazine, atrazine and ametryn (triazines herbicides) and benomyl (benzimidazol fungicide). The results were compared with similar commercial materials. Reversed-phase high-performance liquid chromatography (RP-HPLC) using a Purospher RP-18 5 μm column and ACN: 0.01% aqueous NH_4OH, pH 8.4 (35:65, v/v) as mobile phase, at 0.7 ml min^{-1}, with 235 nm UV detection, was used for separation and quantification of the pesticides. Method validation was performed at three fortification levels (100, 200, 1000 μg l^{-1}). Limits of detection and quantification show that the methods developed can be used to detect the pesticides in concentrations below the maximum residue levels (MRL) established by the Codex Alimentarius, USA, European Union and Brazilian legislations. The results showed that aminopropyl materials have a better performance than the octadecyl sorbents. Laboratory-made materials give results similar to commercial sorbents, with recoveries and precisions in agreement with directives for method validation in residue analysis.

Keywords: Sample preparation; Solid-phase extraction; Multiclass pesticide analysis; Tomatoes; Method validation

1. Introduction

Many pesticides used in agriculture have toxic effects to the environment and to living organisms when applied improperly. Analytical techniques such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) are widely used to monitor the presence of these compounds in water, soils, foods, fruits and vegetables.

Solid-phase extraction (SPE) is one of the most used techniques for sample preparation prior to analysis by the chromatographic procedures. SPE is used for environmental, food, pharmaceutical and biological applications [1–3] and has many advantages over traditional liquid–liquid extraction, such as the use of smaller volumes of organic solvent, ease of automation, lower cost and reduced volumes of toxic residues. SPE is used mainly to remove interferences, for pre-concentration and for sample storage and transport. Most sorbents used in SPE are bonded phases having C_{18} on silica. Organochlorosilanes and organoalkoxysilanes have been used as silylating agents for the preparation of bonded phases [4], and the stability of the ≡Si–O–Si≡ bonds formed between the silylating agents and the hydroxyl groups on the silica surface is the main advantage of these phases [5]. However, this method has some limitations, such as: high reagent cost, time consuming synthesis procedure, use of toxic solvents and

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reagents and the need for an inert atmosphere and high temperatures to carry out the process. An interesting alternative, successfully used to prepare several HPLC stationary phases, such as, poly(methyloctylsiloxane) (PMOS) immobilized onto zirconized silica [6,7], titanium-grafted silica [8,9] and pure silica [10–12], is the substitution of the chemical reaction by depositing a polymer on the support and then immobilizing the polymer using a thermal treatment or gamma irradiation. SPE sorbents based on poly(methyloctadecylsiloxane) (PMODS) immobilized onto silica by gamma radiation or thermal treatment have been described [13–15] and applied for the pre-concentration of pesticides in grapes, water and urine samples, respectively. Some important advantages of this procedure are good performance, lower cost, simplicity and reduction of toxic residues. Aminopropylsilicas are polar phases that exhibit both polar and non-polar interactions [3]. These materials act as normal phase sorbents or weak anion-exchangers and have also been used in reversed-phase applications [16].

The monitoring of multiresidues of pesticides in fruits and vegetables is very important because it involves public health, environmental monitoring and foreign trade aspects. Several recent papers have reported advances in this field [15,17–23].

In this work, a HPLC method multiresidue determination of six pesticides used on tomatoes was developed and validated. Silica-based C_{18} (octadecyl) and NH_2 (aminopropyl) SPE sorbents, made in our laboratory [13,15] were used for sample preparation.

2. Experimental

2.1. Chemicals and materials

The solvents and reagents used for sorbents development, sample preparation and chromatographic analyses (*n*-hexane, methanol and petroleum ether, from Mallinckrodt (Rio de Janeiro, Brazil) acetonitrile (ACN) and dichloromethane, from Mallinckrodt or Merck (Darmstadt, Germany); *n*-pentane from Merck and ammonium hydroxide (NH_4OH) from Synth, (Diadema, Brazil) were all HPLC or analytical reagent grade, as appropriate. The solvents used to prepare the mobile phases were filtered using a 0.45 μm poly(vinylidene) fluoride (PVDF) Millipore membrane (São Paulo, Brazil). Milli-Q water from Millipore (Bedford, MA, USA) was used throughout.

The pesticide standards atrazine (97.7%), simazine (98.3%) and ametryn (96.8%) were obtained from Novartis (Basel, Switzerland), tebuthiuron (99.8%) was acquired from Supelco (Bellefonte, PA, USA) and diuron (99.3%) and benomyl (99.1%) were obtained from Du Pont (Paulínia, Brazil). Standard stock solutions of these pesticides were prepared in methanol.

The silicas for preparing the SPE materials were from Fluka (Buchs, Switzerland) or ACROS (Geel, Belgium); particle size 0.035–0.070 mm (200–400 mesh) with 6 nm pore size, while the polymers poly(methyloctadecylsiloxane) and aminopropyl terminated poly(dimethylsiloxane) (NH_2Pr-PDMS) (Fig. 1) were from United Chemical Technologies (Bristol, USA). The commercial cartridges for SPE were Supelco (LC-NH_2 and LC-18) and Merck (LiChrolut NH_2 and LiChrolut RP-18).

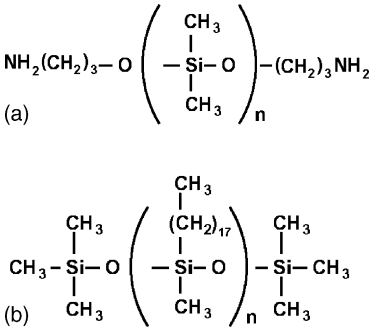

Fig. 1. (a) Aminopropyl-terminated poly(dimethylsiloxane)—NH_2Pr-PDMS and (b) poly(methyloctadecylsiloxane)—PMODS.

The pesticide-free tomato samples produced through organic agriculture were obtained in a local supermarket.

Before all the sample preparation procedures, the laboratory glassware was washed with Extran (Merck).

2.2. Chromatographic instrumentation and conditions

The HPLC system consisted of a Waters (Milford, MA, USA) 510 pump, a SSI (State College, PA, USA) 3XL injector with a 10 μl loop and a Waters UV–vis absorbance detector (Model 481). Data acquisition and treatment was performed by ChromPerfect software, version 3.5 from Justice Innovations (Denvile, NJ, USA). A Purospher RP-18 5 μm column (125 mm × 3 mm i.d.) from Merck and a similar pre-column (4 mm × 4 mm i.d.) were used for the separations. The mobile phase was ACN: 0.01% aqueous NH_4OH, pH 8.4 (35:65, v/v) at a flow rate of 0.7 ml min^{-1} with UV detection at 235 nm. All measurements were carried out at room temperature.

2.3. Preparation and characterization of SPE cartridges

Preparation and characterization of the solid phases was described in an earlier paper [15]. After preparation, the cartridges were made using 0.5 g of sorbent packed into 5 ml polypropylene syringes, retained by two polyethylene frits (20 μm pore size).

2.4. Sample preparation

Spiked samples at three levels (100, 200 and 1000 μg kg^{-1}) were prepared by adding 100 μl of standard solutions of the pesticides to 5 g of pesticide-free tomato samples and mixing thoroughly in a blender. The lowest spiking level was chosen to be close to the Codex Alimentar-

ius [24], European Union [25], United States Environmental Protection Agency (US-EPA) [26] and Brazilian ANVISA [27] Maximum Residue Level (MRL) for these pesticides in fruit and vegetables.

The procedure for the aminopropyl SPE sample preparation was adapted from Hiemstra et al. [28] and is briefly summarized as follows. For solid-phase extraction with laboratory-made aminopropyl or commercial NH_2 cartridges, 7 ml of acetone were added to the sample/standard mixture and homogenized in a vortex mixer (Phoenix, Araraquara, Brazil; model A-250) for 30 s. Seven millilitres of dichloromethane and 7 ml of petroleum ether were then added and the mixture was homogenized for another 30 s. The mixture was then centrifuged for 15 min at 8000 rpm (Fisher Scientific centrifuge, Pittsburgh, PA, USA) and the organic layer was decanted and concentrated under nitrogen. The residue was redissolved in 1 ml of dichloromethane and placed on a 500 mg aminopropyl cartridge, previously conditioned with 2 ml of dichloromethane. The cartridge was eluted in a 12-port SPE vacuum manifold (Supelco Visiprep) with two portions of 3 ml each of dichloromethane:methanol (99:1, v/v), and the eluent was concentrated to dryness and redissolved in 2 ml of methanol for HPLC determination.

Solid-phase extraction with the laboratory-made octadecyl and commercial C_{18} cartridges was adapted from a procedure described by Torres et al. [29]. Here, 20 ml of acetone:water (1:1, v/v) were added to the spiked tomatoes. The sample was mixed thoroughly for 15 min by sonication (Thornton, model T14). The mixture was centrifuged for 15 min at 8000 rpm and 20 ml of water were added to the liquid phase. The 500 mg cartridges were previously conditioned with 5 ml of methanol and 5 ml of water, before applying the sample. The cartridge was eluted in the vacuum manifold with 10 ml of dichloromethane and the eluent was concentrated to dryness and redissolved in 2 ml of methanol for injection into the chromatograph.

2.5. Method validation

Method validation was carried out using parameters proposed by the ICH directives [30,31].

Stock solutions of each pesticide were prepared in methanol at concentrations of 100 $\mu g\,ml^{-1}$ and stored at 4 °C. The solutions for calibration and fortification were prepared in ACN:water (1:1, v/v). The analytical curves were made using six different concentrations (50 $\mu g\,l^{-1}$ to 5 $mg\,l^{-1}$) for each analyte, with three replicates each. For recovery and precision evaluations, samples were spiked at three levels: 100, 200 and 1000 $\mu g\,kg^{-1}$.

The limits of detection (LOD) and quantification (LOQ) were calculated based on the standard deviation of the response and on the slope of the analytical curve [31]. Accuracy was determined as percent recovery, at three different fortification levels. Precision was evaluated in terms of repeatability and intermediate precision, also using three different fortification levels.

Table 1
Analytical curve parameters

Pesticide	a (intercept)	b (slope)	r	Linearity ($\mu g\,l^{-1}$)
Benomyl	3302.22	35.8106	0.99675	50–50,000
Tebuthiuron	2007.40	33.3612	0.99872	50–10,000
Simazine	−828.742	26.4942	0.99977	50–50,000
Atrazine	−1632.36	28.5347	0.99905	50–10,000
Diuron	−2938.27	63.6808	0.99975	50–50,000
Ametryn	−5083.03	74.3967	0.99949	50–10,000

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

3. Results and discussion

3.1. Selectivity

Figs. 2 and 3 shows chromatograms of the spiked tomato extracts, while Fig. 4 shows a similar sample, excluding the SPE purification steps, demonstrating the selectivity of the method. In a general way, these figures give a clear picture of the selectivity of the chromatographic system as well as the purification step. Fig. 2a shows the efficiency of SPE purification and Fig. 2d indicates the presence of matrix components at around 1.5 min, which causes low recovery of benomyl in NH_2 laboratory-made cartridges. Fig. 3 indicates that C_{18} cartridges give a good selectivity, but with a poor recovery.

3.2. Calibration and linearity

The linear regression ($y = a + bx$) parameters for method calibration are presented in Table 1. The analytical curves were obtained over three orders of magnitude and their linearities were evaluated by means of the ratio between signal (S) and concentration (Q), defined by $(S_i/Q_i) = (S_i–a)/Q_i$, where the signal/concentration ratio for the ith point of the analytical curve (S_i/Q_i), is calculated from the signal, S_i, at the corresponding concentration, Q_i, and the intercept of the analytical curve. Based on IUPAC recommendations, points were considered to be in the linear range if their (S_i/Q_i) values did not differ by more than 5% from the slope [32].

In the absence of random errors, i.e. with $r = 1$, and within the linear range, it can be shown that $(S_i/Q_i) = b$, where b is the slope of the curve, for all pairs of experimental values used to construct the curve. In the presence of random errors ($r < 1$), the real situation in the most experimental conditions, and within the linear range, $(S_i/Q_i) \approx b$. If $(S_i/Q_i) < b$ or $(S_i/Q_i) > b$, then the ratio is assumed to be out of the linear range.

3.3. LOD and LOQ

In this study, LOD and LOQ were determined according to the definitions of ICH [30,31]. The results of LOD and LOQ, before and after pre-concentrations, are presented in Table 2, showing LOQ after pre-concentration lower than 100 $\mu g\,kg^{-1}$, satisfying the European, US-EPA, Brazilian and Codex Alimentarius MRL. Considering these results, the method is adequate to determine these pesticides in tomatoes.

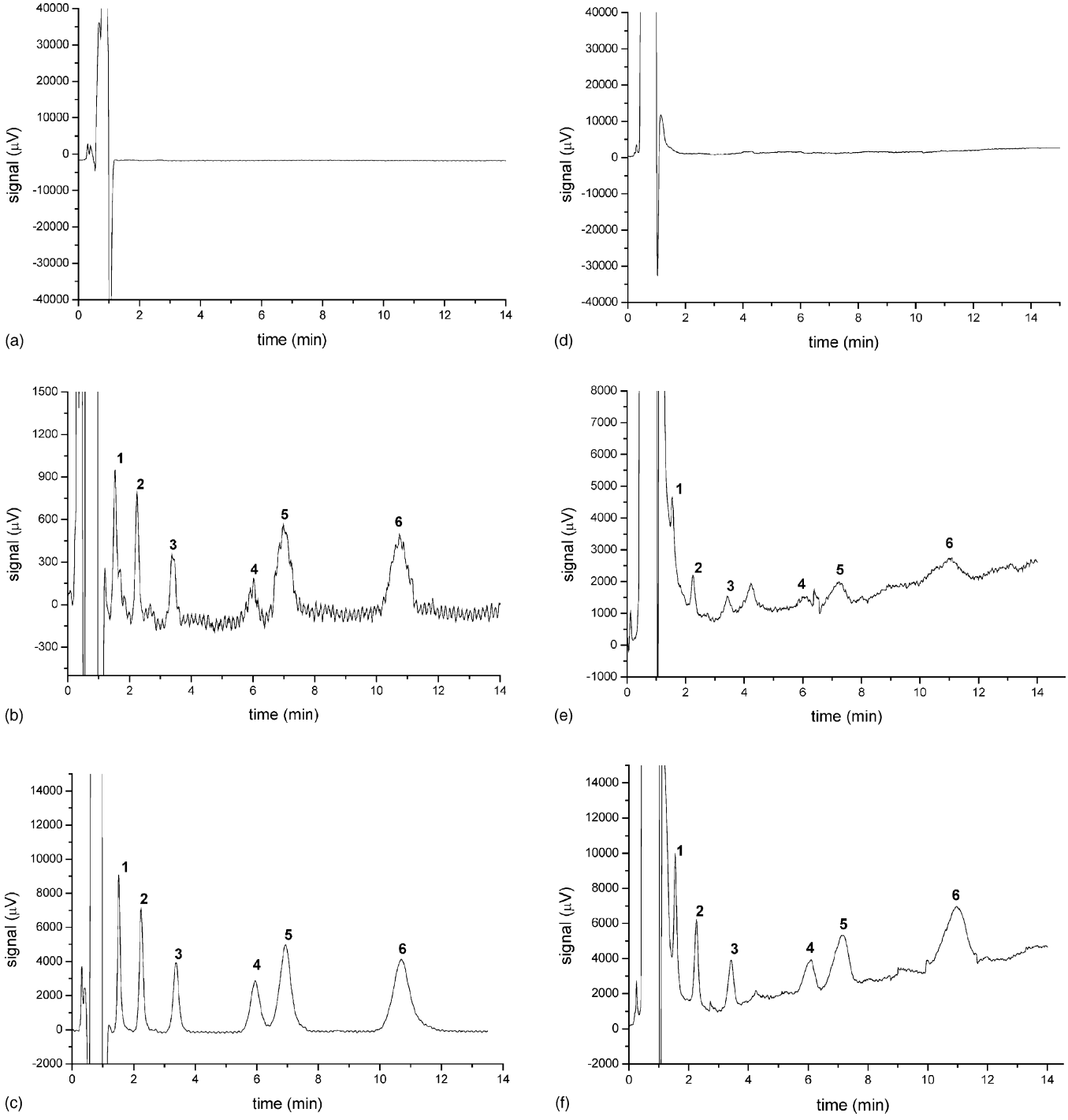

Fig. 2. Chromatograms of the extracts from tomatoes obtained using a NH_2 commercial cartridge: (a) blank and spiked at fortification levels; (b) F1 (100 μg kg^{-1}); (c) F3 (1000 μg kg^{-1}); a NH_2 laboratory-made cartridge (d) blank, spiked at fortification levels; (e) F2 (200 μg kg^{-1}); and (f) F3 (1000 μg kg^{-1}). Chromatographic conditions: injection volume: 10 μl; column: Purospher RP-18 (125 mm × 3 mm), with pre-column (4 mm × 4 mm); mobile phase ACN: 0.01% aqueous NH_4OH, pH 8.4 (35:65, v/v); flow rate: 0.7 ml min^{-1}; detection: 235 nm. Pesticides: (1) benomyl; (2) tebuthiuron; (3) simazine; (4) atrazine; (5) diuron; and (6) ametryn.

3.4. Recovery and precision (repeatability and intermediate precision)

Table 3 shows the recoveries (*R*) and precisions (repeatability and intermediate precision) for the methods developed, using laboratory-made sorbents and similar commercial sorbents for tomatoes spiked at several different levels. These parameters were calculated in agreement with the ICH definitions [31]. Considering the acceptability criteria to be recoveries between 50 and 120% with precisions up to 15%

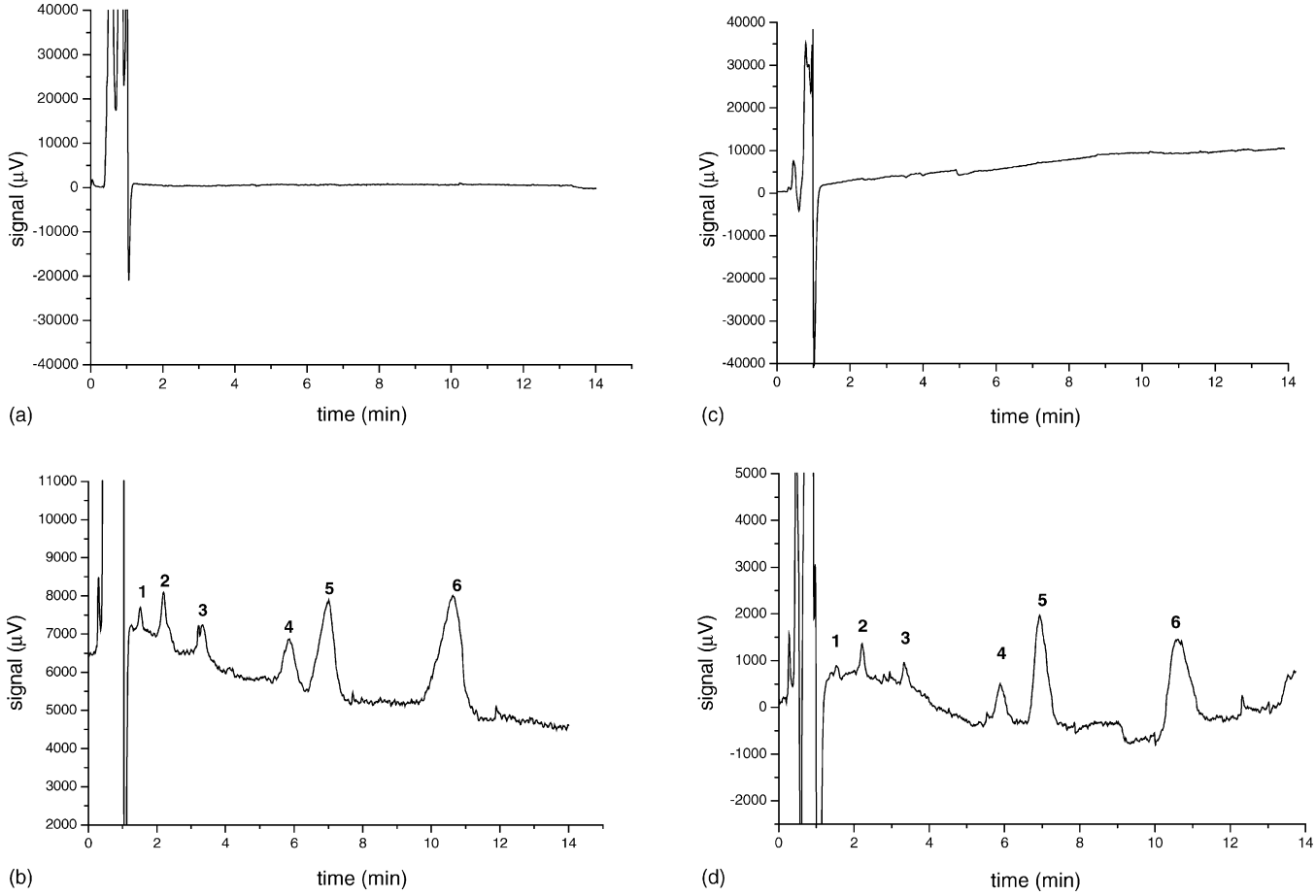

Fig. 3. Chromatograms of the extracts from tomatoes obtained using a C_{18} commercial cartridge: (a) blank and spiked at fortification level; (b) F3 (1000 μg kg^{-1}); and C_{18} laboratory-made cartridge: (c) blank and spiked at fortification level; and (d) F3 (1000 μg kg^{-1}). Chromatographic conditions and peak identification as in Fig. 2.

[33], cartridges with amino-based material generate better results than the octadecyl sorbents.

The methodology using commercial NH_2 cartridges presents good recoveries for all pesticides at concentration levels F2 and F3. At fortification level F1, the over-measured recoveries for benomyl, atrazine and diuron are due to the difficultly in peak integration, since the signal/noise ratio for these peaks is low, as can be seen in Fig. 2b; however, for compounds where the peaks are better defined (tebuthiuron, simazine, ametryn) recovery is within acceptable limits. Repeatability and intermediate precision values are good in most of the experiments.

For laboratory-made 40% loaded-NH_2 cartridges, most of the recovery results are within an acceptable interval. Decreases of recoveries at the fortification level F3 suggest a possible overload of the cartridge.

Methodologies using commercial and laboratory-made C_{18} cartridges had poorer performances than did the NH_2 containing devices. Both commercial and laboratory-made C_{18} cartridges presented acceptable recovery results only for

Table 2
LOD, LOQ and MRL values (μg kg^{-1}) from several agencies

Pesticide	LOD	LOQ	LOD*	LOQ*	MRL Brasil/Codex [24,27]	MRL US-EPA [26]	MRL European Union [25]
Benomyl	54	163	22	65	1000	5000	500
Tebuthiuron	45	138	18	55	n.d.	n.d.	n.d.
Simazine	37	113	15	45	n.a.	n.d.	n.a.
Atrazine	36	108	14	43	n.d.	n.d.	100
Diuron	36	110	14	44	n.d.	n.d.	n.d.
Ametryn	71	214	28	86	n.d.	n.d.	n.d.
Other pesticides	–	–	–	–	>500	>200	>50

n.d.: not defined (MRL not defined), n.a.: not authorized (use of the pesticide not authorized).
* LOD and LOQ after 2.5-fold pre-concentration.

Table 3
Recoveries ($n = 6$) and precision (repeatability, $n = 6$, and intermediate precision, $n = 3$) for pesticides in tomatoes; fortification levels: F1 (100 μg kg^{-1}), F2 (200 μg kg^{-1}) and F3 (1000 μg kg^{-1}) using commercial and laboratory-made (40% polymer load) aminopropyl cartridges and commercial and laboratory-made (40% polymer load) octadecyl cartridges

	Recovery (%)			Repeatability (%)			Intermediate precision (%)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3
Commercial NH_2 cartridges									
Benomyl	127	106	78	9.3	10	5.2	4.6	2.4	6.3
Tebuthiuron	117	99	80	8.2	10	2.8	3.8	0.7	2.3
Simazine	106	81	94	20	32	8.8	16	41	10
Atrazine	127	89	101	20	14	8.6	2.4	0.5	8.7
Diuron	132	96	84	15	5.0	5.	9.1	4.2	1.4
Ametryn	116	102	89	16	12	11	5.1	5.8	14
Laboratory-made NH_2 40% load cartridges									
Benomyl	59	60	45	21	15	10	26	3.7	1.2
Tebuthiuron	82	67	48	19	15	7.7	5.8	13	2.0
Simazine	99	68	57	21	27	7.6	16	18	7.8
Atrazine	96	86	57	31	27	14	7.1	6.9	14
Diuron	67	52	57	15	31	13	13	24	8.3
Ametryn	83	70	64	21	36	26	8.8	32	31
Commercial C_{18} cartridges									
Benomyl	49	29	10	6.6	9.2	22	1.0	6.1	27
Tebuthiuron	59	32	17	20	15	21	12	8.7	1.4
Simazine	55	34	18	24	27	38	3.4	25	23
Atrazine	55	40	39	24	33	15	13	12	0.6
Diuron	53	40	39	8.2	32	34	0.4	11	4.2
Ametryn	61	57	55	25	55	22	5.4	62	19
Laboratory-made PMODS cartridges									
Benomyl	63	29	6.0	14	7.3	12	14	39	11
Tebuthiuron	65	29	10	19	20	16	21	1.2	16
Simazine	69	35	10	20	52	20	9.6	35	22
Atrazine	64	70	18	21	33	26	16	26	12
Diuron	63	46	27	18	12	32	3.0	17	27
Ametryn	61	37	28	17	27	42	10	5.1	49

the F1 level. Laboratory-made C_{18} cartridges give higher recoveries than commercial cartridges.

The low recoveries obtained without purification (Table 4) indicate the presence of matrix components, which were not properly extracted.

Conventional aminopropyl sorbents are on the borderline between polar (normal phase) and ionic exchanger materials. They can act as normal phase sorbents for extraction of polar compounds, phenolic pigments, drugs and metabolites, as weak anionic exchanger for carbohydrates, weak anions and organic acids and have also shown reversed phase characteristics [16].

Table 4
Recoveries (%) for pesticides in tomatoes at fortification level F3 (1000 μg kg^{-1}) without the SPE step

Pesticide	Recovery (%)
Benomyl	60
Tebuthiuron	36
Simazine	35
Atrazine	41
Diuron	34
Ametryn	36

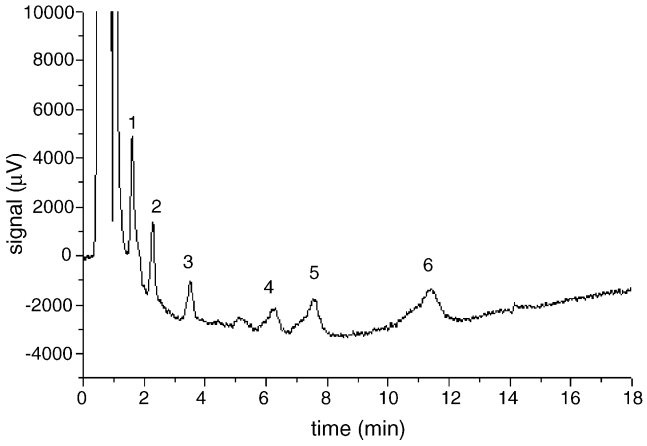

Fig. 4. Chromatogram of tomatoes spiked at fortification level F3 (1000 μg kg^{-1}), without the SPE step. Chromatographic conditions and peak identification as in Fig. 2.

Our results indicate that the laboratory-made NH_2 sorbents also behave as a type of mixed-mode sorbent: possessing apolar characteristics due to the dimethylsiloxane chains and also polar characteristics because of the aminopropyl

terminations, presenting both polar and apolar interactions and adapting well to different chemical environments.

4. Conclusions

The results show that aminopropyl materials have a better performance than the octadecyl sorbents. Laboratory-made materials give results similar or better than commercial sorbents, with recoveries and precisions in agreement with the directives for method validation in residue analysis.

The NH_2-type material, prepared from an aminofunctional siloxane polymer immobilized on a silica surface, presents a fast, easy and effective procedure to obtain silica-based NH_2-type sorbents for use in SPE, with potential for many sample preparation methodologies. The performance of the laboratory-made 40%-loaded NH_2 materials was similar to the commercial cartridges and can be attributed to its mixed-mode sorbent effect, giving a solid phase that can act as a normal phase, a reversed phase and a weak anion exchanger.

New materials for solid-phase extraction were tested in the multiclass analysis of pesticides in tomatoes. The analytical methodologies were validated and presented satisfactory results. These methodologies involved two different SPE modes (clean-up and pre-concentration), using both commercial and laboratory-made materials.

The limits of quantification show that the methods developed can be used to detect the pesticides at concentrations below the Maximum Residue Levels established by the Codex Alimentarius and in Brazilian legislation.

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