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Analytical method validation for the evaluation of cutaneous occupational exposure to different chemical classes of pesticides

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Abstract

In occupational exposure to pesticides, validated methodologies are available only in regards to homogeneous chemical classes of substances and the inhaling exposure, neglecting the cutaneous one that, especially in agriculture, represents an important route of absorption. An analytical methodology for the simultaneous quantification of different chemical classes of pesticides by using *pads* as environmental matrix and GC–MS/SIM as detection method was developed and validated. The extraction step of analytes from pads was optimized by comparing analytes recovery percentages obtained with different extraction solvents. High recoveries were obtained with ether and, above all, with acetonitrile. Validation experiments following the Food and Drug Administration Guidelines were carried out.

Keywords: Cutaneous exposure; Analytical method validation; Pesticides; Pads; Selected ion monitoring

1. Introduction

Pesticides with different chemical nature as well as with different functionality – such as organonitrogenous aliphatic and/or aromatic compounds, carbamate and thiocarbamate pesticides, organophosphorous compounds and piretroids – are used as fungicides, acaricides, insecticides, herbicides, etc., in soils and cultivations treatments in order to increase the production of agricultural and food products [1].

The absorption of pesticides by humans implies numerous health effects, which vary according to the chemical properties of different phytodrugs, and go from respiratory and cutaneous irritations to serious nephropathy and death due to carcinogenic diseases [2–6]. As a consequence, numerous methodologies, aimed toward the protection of consumers and general population, have been developed for pesticide quantification in food and environment [7–17]. In contrast, there are less analytical techniques specifically designed for the healthcare of workers exposed to pesticides [18–23].

In occupational exposure, phytodrugs are mainly absorbed by inhalation and through the skin; the gastroenteric absorption is usually small and it is principally due to the deglutition of particles at the level of the first respiratory tract. In particular, in agriculture, various authors agree to attribute less importance to the inhaling contribution (aerosol and vapours) with respect to the dose absorbed by dermal contact, except for fumigants, because of their volatility [24].

Nevertheless, analytical methodologies suggested by International Agencies, such as OSHA, NIOSH, EPA, mainly propose sampling methods with resins and filters, followed by HPLC-UV or ECD as detection methods. These techniques are suitable to put in evidence the inhaling exposure, neglecting the dermal one. Besides, methods from International Agencies do not include the possibility of the simultaneous determination of different classes of substances [25–27].

In contrast, various monitoring studies can be found in literature, both for the evaluation of inhaling and cutaneous exposure and for the simultaneous determination of more substances

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[28–33]. Nevertheless, while international agencies suggest methodologies that have been partially or fully validated, analytical methods available in literature do not necessarily follow any validation protocol, which is an indispensable requirement to assure data reproducibility and comparison [34]. In particular, despite the widely recognized high sensitivity and specificity levels, on the international scene there are few validated methods involving mass spectrometry as detection method in the evaluation of occupational exposure to pesticides [28,35]. Within this context, a national (Italian) research project (PON N. 12777, MIUR) has been developed, based on two objectives: (1) the optimization of analytes extraction step from pads, which have to be applied for the evaluation of potential dermal exposure, followed by GC–MS analysis. Different extraction solvents were used and analytes recovery percentages were calculated and compared; (2) the validation of the whole analytical procedure, by carrying out the experiments suggested by the latest FDA Guidelines [34].

With this aim the study was carried out on 12 substances belonging to different chemical classes of pesticides; among those used in the rural areas investigated were: chlorpyriphos, chlorpyriphos methyl, λ-cyhalothrin, cypermethrin, dimethoate, dimethomorph, hexaconazole, hexythiazox, lindane, metalaxyl, propargite and tebufenpyrad (Table 1).

2. Experimental

2.1. Chemicals, supplies, instrumentation

All analytical reference standard pesticides were obtained from Sigma–Aldrich (St. Louis, MO, United States). Whatman 2 fiber paper pads (46 × 57 cm^2) and HPLC grade solvents were purchased from Whatman International Ltd. (Maidstone, England) and Carlo Erba (Milan, Italy), respectively. GC–MS analyses were carried out by using a Focus GC interfaced with a single quadrupole mass spectrometer DSQ, equipped with an autosampler AS 3000, operated using Xcalibur software version 1.2 (Thermo-Electron Corporation, Waltham, MA, United States). A DB5-MS (15 m × 0.25 mm × 0.25 μm) capillary column (J & W Scientific, Falson, CA, USA) was used for gas chromatographic separations.

2.2. Analytical conditions

Samples (injected volume, 1 μl) were introduced into the gas chromatograph injector held at 260 °C. The temperature program used was the following: 75 °C for 2 min, 75–300 °C at 15 °C min^{-1}, 300 °C for 3 min, equilibration time was 0.5 min. Helium was used as carrier gas with a constant flow of 1.2 ml min^{-1}.

Mass spectrometric data were obtained under the following conditions: electron ionization, 70 eV; source temperature, 250 °C; transfer line, 260 °C; emission current, 100 μA.

Mass spectra were first obtained in full scan mode (range of acquisition, 50–450 *m/z*), in order to define analytes retention times and to identify qualifying ions that had to be used in selected ion monitoring mode. Following analyses were performed in SIM by dividing the chromatographic run in different segments, each one centered on the analytes retention time. For each segment, two to four characteristic ions related to each substance were acquired. Selected Ions and retention times (t_r) of each investigated analytes are reported in Table 1.

2.3. Preparation of stock solutions, matrix-based calibration standards and quality control samples

Stock solutions of each pesticide were prepared in acetonitrile at a concentration of 1 μg μl^{-1}. Tebufenpyrad and chlorpyriphos methyl solutions were not diluted, and these pesticides were used as internal standards, according to the performed experiment. On the contrary, two mixtures of the other pesticides were obtained by diluting with acetonitrile, with concentrations of 25 ng μl^{-1} and 80 ng μl^{-1}. The first pesticides mixture was used for the GC–MS characterization and for the optimization of the extraction procedure; the second one was further progressively diluted (1:3) with acetonitrile to obtain other six working

Table 1
Investigated analytes: classification, retention times and selected ions

Substances	Chemical class[a]	CAS	Molecular formula	t_r (min)	Selected Ions (*m/z*)
Chlorpyriphos	Organophosphorus	2921-88-2	$C_9H_{11}Cl_3NO_3PS$	12.85	314.1, 316
Chlorpyriphos methyl	Organophosphorus	5598-13-0	$C_7H_7Cl_3NO_3PS$	12.23	279.1, 285.8, 287.9, 289.9
Cyhalothrin	Pyrethroid	91465-08-6	$C_{23}H_{19}ClF_3NO_3$	16.42	181.2, 197.1, 208.2
Cypermethrin	Pyrethroid	97955-44-7	$C_{22}H_{19}Cl_2NO_3$	17.70	163.1, 165.0, 181.1, 209.2
Dimethoate	Organophosphorus	60-51-5	$C_5H_{12}NO_3PS_2$	11.13	87.1, 125.0, 229.0
Dimethomorph	Morpholine	110488-70-5	$C_{21}H_{22}ClNO_4$	19.58	301.1, 303.1, 387.3
Hexaconazole	Azole	79983-71-4	$C_{14}H_{17}Cl_2N_3O$	14.08	214.1, 216.1, 256.1
Hexythiazox	Carboxamide[b]	78587-05-0	$C_{17}H_{21}ClN_2O_2S$	13.77	156.1, 184.2, 227.1
Lindane	Organochlorine	58-89-9	$C_6H_6Cl_6$	11.44	181.0, 183.0, 219.0
Metalaxyl	Xylylalanine	57837-19-1	$C_{15}H_{21}NO_4$	12.42	160.1, 206.2
Propargite	Organosulfite[c]	2312-35-8	$C_{19}H_{26}O_4S$	15.38	173.2, 201.0, 350.3
Tebufenpyrad	Pyrazole	119168-77-3	$C_{18}H_{24}ClN_3O$	15.97	276.0, 318.1, 333.1

[a] Chemical classification from Pesticide Action Network Pesticides Database [36]. When pesticides were not registered in this database, molecular classes were assigned basing on their molecular structure and the chemical name is reported.
[b] *trans*-5-(4-Chlorophenyl)-*N*-cyclohexyl-4-methyl-2-oxothiazolidine-3-carboxamide.
[c] (2-(4-*tert*-butylphenoxy)cyclohexyl 2-propynyl sulfite.

solutions, with concentrations of 0.11, 0.33, 0.99, 2.96, 8.87 and 26.6 ng μl^{-1}.

Seven matrix-based calibration standards were obtained by adding 500 μl of each working solution to seven $7 \times 7\,cm^2$ fiber paper pads, in order to obtain analytes concentrations, with respect to pad surface areas of 1.1, 3.4, 10.1, 30.2, 90.5, 271.4 and 816.3 ng cm^{-2}. Equal volumes (6.25 μl) of tebufenpyrad 1 μg μl^{-1} solution (internal standard) were also added to each pad. Acetonitrile was allowed to evaporate by keeping pads for 1 h at 25 °C, and then samples were stored at −20 °C. Similarly, 12 quality control samples at four concentration levels (0.20, 1.26, 7.08 and 30.30 ng μl^{-1}, corresponding to 2.04, 12.86, 72.24 and 309.39 ng cm^{-2} in matrix) were independently prepared, treated and analysed, and results were used for the evaluation of methodological accuracy and precision.

2.4. Analytes extraction procedure

The optimization of analytes extraction from pads was performed by using 25 ng μl^{-1} pesticides mixture solution. 500 μl was added to fiber paper pads. Three extraction parameters were considered: the most suitable extracting solvent, various sonication times and variable number of solvent aliquots.

Pads were cut in small pieces and added with 10 ml of six different extracting solvents: ethylacetate, methanol, toluene, acetone, acetonitrile and ether. Then, samples were sonicated and two sonication times were tested (10 and 30 min). The extraction was repeated either two or three times, comparing the analytes recovery percentages obtained by using two or three aliquots of each extracting solvent. In both cases, collected solvent aliquots were dried under nitrogen and residues were solved with 500 μl of acetonitrile. 6.25 μl of the tebufenpyrad stock solution was added before analysis (internal standard) and all the extracting procedures tested were repeated in triplicate. Relative recovery percentages were calculated by comparing the analytical responses (ratio between the chromatographic peaks areas of analyte and of the internal standard) of pads extracted analytes with respect to unextracted standards, which represent 100% recovery.

Relative recovery percentages were also calculated for tebufenpyrad by extracting pads added only with 500 μl of a 25 ng μl^{-1} tebufenpyrad acetonitrile solution and by using chlorpyriphos methyl as internal standard.

After deciding all extraction parameters, a final analytes extraction procedure was established, and calibration standards and quality control samples were treated by using three acetonitrile aliquots of 10 ml each with a sonication time of 10 min.

2.5. Validation procedure

Validation of the method was performed according to the latest FDA guidelines [34].

2.5.1. Linearity, accuracy and precision

Matrix-based calibration curves were obtained for each analyte. Each calibration curves consisted of two zero samples (pads containing only the internal standards) and seven non-zero matrix-based calibration standards (pads added with known analytes amount as well as with the internal standard). GC–MS/SIM peak areas of each analyte with respect to those of the internal standard were measured (areas ratio = analytical response) and given as a function of the concentration. A linear fit was used to obtain calibration curves equations. Each calibration curve was prepared in triplicate, and the response linearity was evaluated by calculating correlation coefficients (r^2). Back-calculated calibration concentrations were also determined, as well as the accuracy and precision of the matrix-based calibration standards, i.e. the percentage deviation of the so-determined concentrations from nominal ones (Acc%) and the coefficients of variation (CV), respectively. In order to assure the reliability and reproducibility not only for calibration curves but also for the whole methodology, accuracy and precision must also be evaluated by analysing quality control samples. Four concentration levels were considered (see above) and analysed together with a set of matrix-based calibration standards, independently prepared from the quality control samples. The experiment was repeated three times.

According to the FDA guidelines, both the accuracy and precision of matrix-based calibration standards and of quality control samples can be accepted if deviations are within ±15%, except at the lower limit of quantification (LLOQ) level, where deviations within ±20% are accepted. Analogously, CVs must result <20% at LLOQ level and <15% at all other concentration levels.

2.5.2. Specificity and sensitivity

Six blank samples (pads without the addition of any pesticide) were analysed according to the procedure previously described, in order to evaluate method specificity. The absence of interfering compounds, characterized by ions at m/z values equal to those of the investigated analytes and eluting at the same analytes retention time, was verified.

The limits of detection (LOD) and of quantification were determined by analysing six zero samples. GC–MS/SIM peak areas detected at the retention times of the analytes of interest were measured; the ratios with respect to chromatographic peak area of internal standard were calculated and calibration curves equations were used to determine 'virtual' mean analyte concentrations (C_{Mi}, i = analyte). LODs and LLOQs were, respectively, defined as: LOD = C_{Mi} + 3SD; LLOQ = C_{Mi} + 5SD (SD, standard deviation).

2.5.3. Recovery

Extraction recovery is defined as the analytical response obtained from an amount of the analyte added to and extracted from the investigated matrix, compared with the analytical response obtained for the true concentration of the pure standard. Recovery experiments should be performed by comparing unextracted standards, which represent 100% recovery, with extracted samples, where analytes should have been added at least at three different concentrations (low, medium, high). Here we considered the whole investigated analyte concentration range. Two calibration curves were calculated: the first one was based on the analyses of matrix-based calibration standards and

the second one was obtained by the GC–MS/SIM analysis of working solutions in acetonitrile. Recovery was calculated by the ratio between the slopes of the two curves. The experiment was repeated in triplicate and standard deviations were calculated.

2.5.4. Stability

Short-term stability was studied by using three working solutions at 15.2, 5.06 and 1.26 ng μl^{-1} containing all the analytes of interest, except chlorpyriphos methyl. An aliquot of these solutions was added with chlopyriphos methyl as internal standard and immediately analysed; the obtained analytical responses represented the 100% stability. Working solutions were again analysed after keeping them for 6 and 24 h at room temperature, while the internal standard solution was stored at −20 °C and added soon before analysis. Each analysis was repeated in triplicate.

Long-term stability was studied. 27 pads were added with equal volumes of the above-reported working solutions to obtain nine matrix-based samples for three analyte concentrations: 155.1, 51.6 and 12.8 ng cm^{-2} (concentration levels: A, B and C, respectively). 3A, 3B and 3C were immediately extracted, added with chlorpyriphos methyl and analysed; the others were stored at −20 °C. After 1 week, 3A, 3B and 3C were unfrozen, extracted, added with the internal standard and analysed. The same procedure was repeated after 1 month.

3. Results and discussion

3.1. Optimization of extraction recovery

During method development step, recovery percentages were calculated in order to choose the better extraction conditions. Table 2 reports recovery percentages of investigated analytes, extracted from pads by using six different solvents, and by repeating the extraction step twice, with a sonication time of 30 min. Internal standards (chlorpyriphos methyl for measuring tebufenpyrad analytical response and tebufenpyrad for all the other substances) were added soon before GC–MS/SIM analyses to measure analytical responses, so that the latter were not influenced by the extraction recovery of internal standards themselves. The choice of the most suitable solvent was based on three criteria: high recovery percentages (Rec%); simultaneous recovery of more substances; lesser standard deviation (SD). Ethylacetate and toluene, followed by methanol, gave the worst results, while high recovery percentages were obtained with ether and, above all, with acetonitrile. Acetonitrile was chosen as extraction solvent because of the third choice criteria;

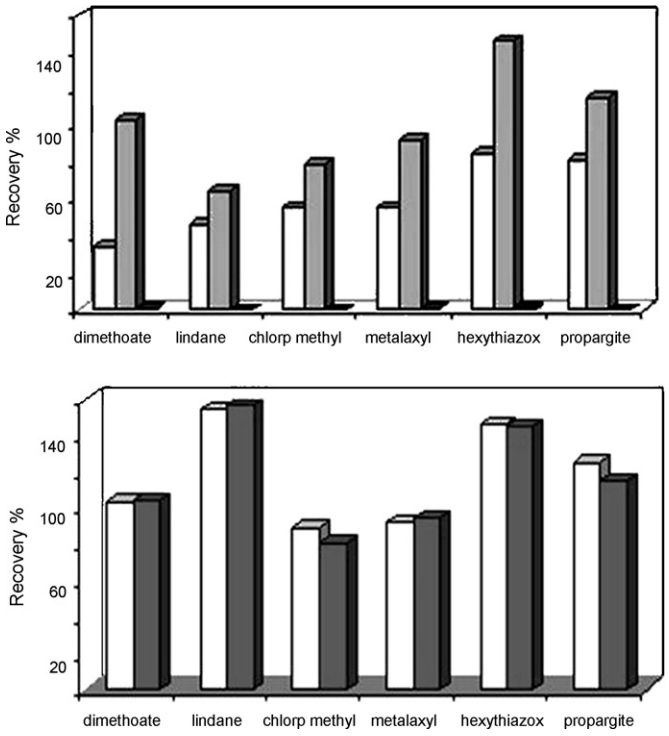

Fig. 1. Average recovery percentages of six pesticides extracted from pads. Top panel: extractions were carried out with constant 10 ml aliquots volume and 30 min sonication time, by varying the number of acetonitrile aliquots used during the extraction; first bar, two aliquots collected and analysed; second bar, three aliquots collected and analysed; third bar, amount of each analyte found in a fourth aliquot, separately analysed after having collected three aliquots. Bottom panel: extractions were carried out by collecting three acetonitrile 10 ml aliquots, and by varying the sonication time: 10 min and 30 min, first and second bars, respectively.

Table 2
Recovery percentages of analytes extracted from pads by using different solvents

	Rec% ± SD					
	Methanol	Acetonitrile	Ethylacetate	Toluene	Acetone	Ether
Chlorpyriphos	74.5 ± 14.2	99.7 ± 7.2	24.4 ± 2.3	77.9 ± 4.8	95.0 ± 4.4	81.3 ± 14.4
Chlorpyriphos methyl	67.8 ± 1.9	92.4 ± 3.2	75.7 ± 4.2	54.6 ± 4.5	87.4 ± 18.1	53.4 ± 6.3
Cyhalothrin	22.1 ± 8.8	92.0 ± 7.4	41.2 ± 3.5	74.4 ± 11.2	77.1 ± 19.8	85.7 ± 8.6
Cypermethrin	64.6 ± 20.2	94.1 ± 4.2	62.8 ± 6.0	82.4 ± 2.4	97.3 ± 7.0	87.2 ± 7.2
Dimethoate	98.4 ± 20.1	110.0 ± 4.9	72.8 ± 36.3	43.6 ± 23.3	103.2 ± 16.1	67.5 ± 10.8
Dimethomorph	118.0 ± 19.8	140.4 ± 12.2	75.7 ± 15.7	72.8 ± 2.0	167.5 ± 29.9	132.4 ± 3.1
Hexaconazole	120.0 ± 7.5	135.9 ± 7.8	128.8 ± 18.5	87.8 ± 16.3	130.1 ± 24.0	122.2 ± 19.2
Hexythiazox	114.6 ± 25.8	132.5 ± 4.6	105.3 ± 4.9	94.7 ± 23.6	139.1 ± 33.6	70.9 ± 7.9
Lindane	54.6 ± 10.0	154.7 ± 6.5	59.8 ± 15.3	25.7 ± 19.6	59.9 ± 14.0	78.4 ± 23.1
Metalaxyl	90.1 ± 4.0	92.4 ± 2.0	78.4 ± 27.7	54.5 ± 20.5	90.4 ± 11.0	60.3 ± 4.7
Propargite	92.0 ± 20.1	122.3 ± 6.8	93.5 ± 7.1	78.9 ± 15.0	104.5 ± 21.4	128.4 ± 19.9
Tebufenpyrad	64.6 ± 10.6	158.4 ± 5.1	65.3 ± 10.8	103.0 ± 21.1	69.4 ± 15.9	135.7 ± 10.3

in fact, recovery SD went from 2 to 17% (mean = 6.9%) for acetonitrile with respect to a mean value of 11.0% when ether was used.

After choosing the most suitable extraction solvent, different number of extractions (solvent aliquots) and sonication times were tested, in order to obtain high analytes recoveries. The obtained results are shown in Fig. 1. As evident from the comparison of bars heights for each of the reported analytes, after two extractions (top panel, first bar), significant amounts of each analyte still remain on the pads, from which they are quite completely removed by a third extraction (second bar), while analytes were absent in the fourth solvent aliquot (third bar). In contrast, analytes were extracted with analogous extent when sonication times were varied from 10 to 30 min (Fig. 1, bottom panel).

3.2. Validation of the analytical method

3.2.1. Linearity

Matrix-based calibration standards, in the range 1.1–816.3 ng cm^{-2} pads, were independently prepared and analysed in triplicate, in three different days. Correlation coefficients of linear fit curves obtained from data were in the range from 0.9973 to 0.9998 for all the investigated analytes. Calibration standards concentrations were back calculated. Deviations from the nominal concentrations and CV values were from |0.02| to |14.0|% and 0.4 to 12.3% for all analytes concentrations levels.

The accuracy and precision of the analytical method were evaluated by analysing quality control samples at four concentration levels (QC_i). The obtained values are reported in Table 3 and resulted within FDA requirements for all the investigated analytes. In particular, the Acc% and CVs values of QC_4 samples – at concentrations close to the LLOQ levels reported below (Table 4) – are <15% in the majority of the examined cases, except for chlorpyriphos methyl, cyhalothrin, lindane and propargite, for which Acc% is within 20% deviation.

3.2.2. Specificity and sensitivity

The analyses of matrix-based blank samples showed that no interfering species were present at the retention time of the investigated analytes, assessing method specificity (Fig. 2). The absence of any significant chromatographic peak, when samples without pesticides were analysed, was confirmed by the chromatographic profiles of zero-point samples, which were used to calculate detection and quantification limits. The obtained results are reported in Table 4. Since FDA Guidelines do not specify the experiments that should be performed in determining LODs and LLOQ, they were calculated as recently reported [37,38], i.e. by measuring the area of chromatographic peaks

Table 3
Quality Control samples: accuracy and precision

	QC_1 (309.39 ng cm^{-2})		QC_2 (72.24 ng cm^{-2})		QC_3 (12.86 ng cm^{-2})		QC_4 (2.04 ng cm^{-2})	
	Acc%	CV	Acc%	CV	Acc%	CV	Acc%	CV
Chlorpyriphos	5.9	3.4	−2.3	5.2	2.0	6.8	9.0	3.9
Chlorpyriphos methyl	−4.4	2.9	−5.4	5.7	1.7	2.2	17.1	4.2
Cyhalothrin	−4.8	3.4	−7.7	2.3	2.6	3.7	16.8	2.9
Cypermethrin	−6.3	2.5	−8.7	3.8	−9.7	5.8	4.3	3.9
Dimethoate	−10.4	2.4	−13.1	1.6	−3.5	0.5	−0.7	3.7
Dimethomorph	−9.3	2.6	−2.4	3.1	1.9	4.2	−6.7	13.9
Hexaconazole	−7.3	2.5	−2.2	1.7	−1.8	4.8	6.5	3.6
Hexythiazox	5.2	4.0	2.3	2.0	3.2	3.1	5.8	3.6
Lindane	−6.3	5.2	−5.4	6.2	−8.7	8.7	15.3	5.0
Metalaxyl	5.5	3.1	10.9	1.0	5.7	0.9	5.8	2.8
Propargite	−6.1	1.4	0.5	1.6	7.7	2.2	17.0	3.7

Table 4
Validation parameters: sensitivity and recovery

	Sensitivity			Recovery
	Signal/noise at 1.1 ng cm^{-2} analyte concentration	LOD (ng cm^{-2})	LLOQ (ng cm^{-2})	Rec% ± SD
Chlorpyriphos	70.9	0.7	1.0	88.0 ± 1.9
Chlorpyriphos methyl	29.4	0.9	1.1	89.7 ± 4.2
Cyhalothrin	4.8	0.9	1.1	115.5 ± 1.7
Cypermethrin	2.6	0.5	0.8	130.4 ± 4.5
Dimethoate	4.1	0.9	1.1	95.0 ± 8.5
Dimethomorph	8.1	0.8	0.9	103.6 ± 0.3
Hexaconazole	9.1	0.8	1.1	97.1 ± 8.1
Hexythiazox	3.0	0.9	1.0	112.1 ± 8.2
Lindane	5.3	0.9	1.1	74.6 ± 2.7
Metalaxyl	9.8	0.4	0.6	84.9 ± 2.8
Propargite	3.9	0.9	1.0	100.1 ± 8.4

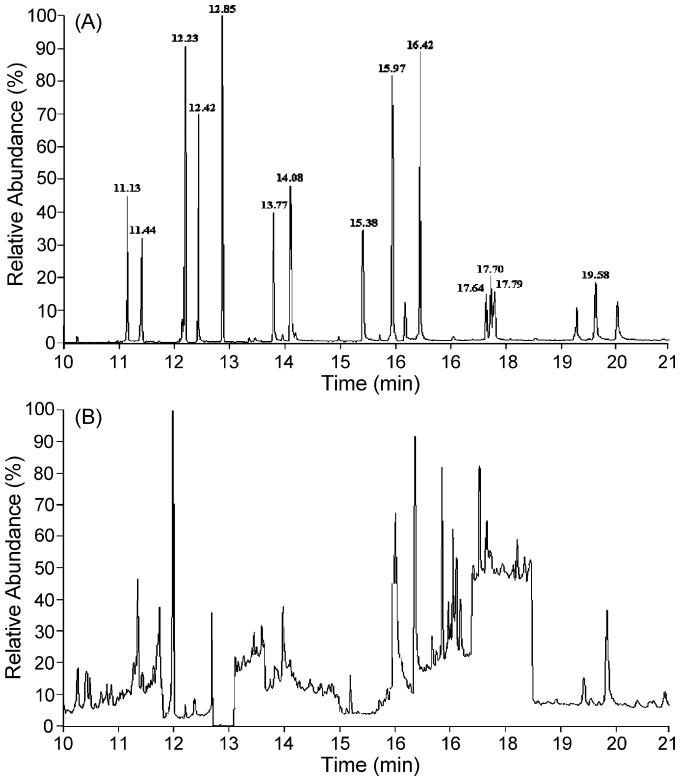

Fig. 2. Selected ion monitoring chromatogram of (A) extracted fortified pad, (B) extracted blank pad.

at analytes retention times. Nevertheless, it could happen that there are no interfering chromatographic peaks eluting at the retention time of interest, as actually occurred during GC–MS analyses carried out in this study. So, the use of this method to determine LOD was actually based on the evaluation of noise, rather than on the lower detectable concentration of analytes. The latter, in fact, could be lost during the various steps of the adopted analytical procedure (storage and extraction), and for this reason they could be not detected even if the analytical noise is low. This is evident when matrix-based samples with analytes concentrations of 1.1 ng cm^{-2} were analysed. In fact, for example, in the case of cypermethrin and hexythiazox, the LLOQs calculated by analysing zero-point samples were 0.8 and 1.0 ng cm^{-2}, respectively. Nevertheless, when samples containing these two phytodrugs at 1.1 ng cm^{-2} were analysed, chromatographic peaks with signal-to-noise ratios lesser than 3 were obtained, so these analytes should not be quantifiable at this concentration level. In contrast, chlorpyriphos and chlorpyriphos methyl still showed quantifiable chromatographic peaks also at 1.1 ng cm^{-2}. That is why, Table 4 also reports signal-to-noise values corresponding to the analysis of matrix-based samples added with analytes concentration of 1.1 ng cm^{-2}.

3.2.3. Recovery

The analytes recovery after the extraction procedure was calculated by analysing and comparing matrix-based samples with respect to working solutions at different concentrations, according to validation protocol. Recovery has to be calculated

Table 5
Short-term and long-term stability

	Short-term stability ± SD (%) calculated on working solutions						Long-term stability ± SD (%) calculated on matrix-based samples					
	(A) 15.2 ng µl^{-1}		(B) 5.06 ng µl^{-1}		(C) 1.26 ng µl^{-1}		(A) 155.1 ng cm^{-2}		(B) 51.6 ng cm^{-2}		(C) 12.8 ng cm^{-2}	
	6 h[a]	24 h[a]	6 h[a]	24 h[a]	6 h[a]	24 h[a]	1 Week[b]	1 Month[b]	1 Week[b]	1 Month[b]	1 Week[b]	1 Month[b]
Chlorpyriphos	101.6 ± 2.0	98.4 ± 0.9	105.4 ± 1.5	100.3 ± 1.2	101.6 ± 2.0	102.4 ± 0.9	93.3 ± 6.6	88.8 ± 8.1	100.1 ± 8.0	77.1 ± 2.3	97.3 ± 7.1	77.7 ± 4.5
Cyhalothrin	96.8 ± 3.6	94.1 ± 5.2	105.4 ± 1.7	103.9 ± 6.9	112.8 ± 5.5	86.0 ± 7.7	93.0 ± 7.2	54.4 ± 0.9	91.4 ± 5.8	54.5 ± 4.2	98.5 ± 1.7	55.5 ± 1.5
Cypermethrin	94.3 ± 4.8	94.9 ± 7.9	103.3 ± 3.4	105.1 ± 9.0	115.1 ± 5.9	97.1 ± 9.7	94.3 ± 8.8	52.0 ± 0.7	93.1 ± 12.5	49.8 ± 3.7	101.1 ± 2.2	47.2 ± 0.7
Dimethoate	92.1 ± 4.0	98.1 ± 3.8	98.3 ± 7.9	99.7 ± 12.3	109.6 ± 4.1	81.3 ± 20.8	111.9 ± 9.4	44.5 ± 0.0	100.6 ± 7.7	42.7 ± 3.1	67.8 ± 10	35.2 ± 2.5
Dimethomorph	144.6 ± 7.7	133.1 ± 7.9	104.6 ± 4.5	93.0 ± 5.6	113.4 ± 6.8	85.9 ± 5.9	94.5 ± 4.2	96.6 ± 4.3	102.1 ± 7.0	89.8 ± 0.5	102.5 ± 3.7	77.7 ± 1.7
Hexaconazole	97.5 ± 0.5	86.8 ± 7.5	107.3 ± 1.0	93.2 ± 9.3	135.4 ± 11.2	90.2 ± 19.5	96.4 ± 6.5	61.0 ± 3.5	93.6 ± 6.1	53.7 ± 0.7	80.8 ± 8.9	45.9 ± 3.7
Hexythiazox	84.4 ± 7.4	101.5 ± 13.3	90.4 ± 9.6	82.6 ± 12.1	117.2 ± 17.9	103.8 ± 19.8	69.5 ± 5.5	44.4 ± 5.6	71.4 ± 4.1	42.7 ± 0.2	41.4 ± 5.5	39.7 ± 1.9
Lindane	94.6 ± 2.5	96.8 ± 8.1	95.6 ± 3.9	93.2 ± 11.0	106.3 ± 3.0	109.4 ± 2.7	95.6 ± 4.7	84.7 ± 7.0	99.4 ± 8.7	74.1 ± 3.4	93.8 ± 8.2	72.2 ± 3.1
Metalaxyl	95.0 ± 3.6	101.4 ± 0.7	102.0 ± 1.9	106.0 ± 2.1	110.1 ± 1.2	106.4 ± 2.6	89.7 ± 6.1	62.7 ± 3.0	92.7 ± 2.5	62.9 ± 1.4	76.5 ± 7.7	55.4 ± 1.4
Propargite	98.2 ± 0.4	88.8 ± 4.5	112.0 ± 0.9	104.0 ± 7.2	119.4 ± 5.5	90.3 ± 8.0	94.2 ± 6.9	58.7 ± 1.3	94.6 ± 8.2	54.7 ± 1.9	83.5 ± 9.7	51.3 ± 2.1
Tebufenpyrad	104.0 ± 1.7	99.3 ± 2.8	107.2 ± 2.5	107.4 ± 1.4	113.7 ± 5.2	97.4 ± 6.0	135.6 ± 4.2	122 ± 7.1	134.2 ± 4.1	99.7 ± 5.4	110.1 ± 2.6	78.9 ± 4.2

[a] Room temperature.
[b] Storage at −20 °C.

following the whole analytical procedure that it refers to; that is why, as analytes were quantified by measuring the areas ratio between analyte and internal standard chromatographic peaks, tebufenpyrad was used as internal standard and analytical responses were registered. The obtained recovery values are reported in Table 4.

3.2.4. Stability

Short-term stability after 6 and 24 h at room temperature was studied to verify if analytes degrade over the course of analyses. Short-term stability can be evaluated by analysing either working solutions or matrix-based samples added with working solutions and kept at room temperature before the extraction step. Since the considered environmental matrix is solid (pads), when analytes were added to the matrix, they were always left at room temperature for at least 1 h, allowing solvent evaporation. Therefore, short-term stability was established by using working solutions and not matrix-based samples. In contrast, long-term stability was studied in order to be sure that analytes present in pads coming from environmental monitoring samplings ("real" samples) do not degrade in the storage conditions before being analysed. Hence, long-term stability was studied on matrix-based samples stored at −20 °C for 1 week and 1 month.

The stability of analytes (expressed as percentage) at room temperature and in the storage conditions was evaluated by comparing the analytical responses, respectively, of working solutions analysed after 6 and 24 h with respect to that of the same solutions immediately analysed, and of samples extracted after 1 week and 1 month with respect to that of samples immediately extracted and analysed.

In both cases, GC–MS analyses were necessarily carried out at different times (hours, weeks or months), during which instrumental conditions (as far as regards tuning and cleaning) could vary, leading to incomparable responses. In order to be sure that the difference of signals was actually due to analyte stability and not to eventual instrumental variations, an internal standard (chlorpyriphos methyl) was added soon before analysis in order to be independent from the investigated stability conditions, and relative analytical responses were measured as area ratios with respect to the internal standard. Table 5 reports the obtained results, showing that analytes are stable at room temperature for 24 h. Samples can be stored at −20 °C for 1 week without a relevant loss of signal, except for hexythiazox, which should be immediately determined. The amount of tebufenpyrad added to pads (127.5 ng cm^{-2}) assures internal standard stability for 1 month; nevertheless, when samples were unfrozen and analysed after 1 month, only low percentages (from 35.2 to 77.7%) of analytes were recovered.

4. Conclusions

An analytical methodology for the simultaneous quantification of different classes of pesticides in pads by GC–MS/SIM is described. Full validation according to the FDA guidelines was performed, and detection levels of ng cm^{-2} (order of magnitude) were reached for all the examined substances, with accuracy and precision levels within FDA requirements.

This method is suitable for the estimation of dermal exposure to pesticides in open air field applications, in particular, when pesticides are applied by spraying [39].

As the cutaneous exposure represents an important route of absorption and as workers are often simultaneously exposed to pesticides with variable chemio-physical properties, the obtained results allow and strongly suggest using a simple and widespread technique as the selected ion monitoring one (that requires a single quadrupole mass spectrometer, nowadays available in the majority of the analytical laboratories) in routine analyses for the evaluation of occupational exposure to pesticides.

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