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Determination of dithiocarbamates and metabolites in plants by liquid chromatography–mass spectrometry☆

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Abstract

A quantitative matrix solid-phase dispersion and liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI–MS) method is outlined for the simultaneous analysis of dithiocarbamates (DTCs) and their degradation products in plants. Compounds analyzed are dazomet, disulfiram, thiram and the metabolites ethylenthiourea and propylenthiourea. The performance of two different sample preparation protocols, the proposed one and other based on solid-phase extraction, as well as, of both atmospheric pressure ionization sources, APCI and electrospray, were compared. The effect of several parameters on the extraction, separation and detection was studied. Dithiocarbamates and metabolites were dispersed with carbograph, eluted with a mixture of dichloromethane–methanol, and then, identified by monitoring the base peak of the spectra corresponding to $[M + H]^+$. The method was validated for avocados, cherries, lemons, nuts, oat, oranges, peaches, rice and tomatoes. Average recoveries varied from 33 to 109%, and relative standard deviation were between 4 and 21% with limits of quantification ranged from 0.25 to 2.5 mg kg^{-1}, except for thiram and disulfiram, which were not recovered from fruits with high acid content. The procedure was applied to the determination of DTCs and their metabolites in fruits, vegetables and cereals taken from different markets of Valencia, Spain.

Keywords: Food analysis; Dithiocarbamates; Organosulfur compounds; Pesticides

1. Introduction

Dithiocarbamates (DTCs) are important organosulfur compounds, which act as inhibitors of metal dependant and sulphydryl enzymes and have a serious consequence on biological systems. They possess variety of applications in agriculture as fungicides, as well as, in the rubber industry as vulcanization accelerators and antioxidants [1]. In this way, DTCs are the main group of fungicides used to control approximately 400 pathogens of more than 70 crops and are registered in all the EU member states and many other countries [2,3]. Ethylenthiourea (ETU) and propylenthiourea (PTU) are the most important transformation products of DTCs suspected to cause various pathogenic effects (e.g., goiterogenic, carcinogenic, mutagenic, teratogenic) [4]. Determination of DTCs subclasses is required for toxicological evaluations since the DTCs and their metabolites differ greatly in their action mechanism [4,5]. In recent years, concern has been expressed on their presence in foods, drinks and environment [5].

The predominant methods for determining DTCs and metabolites are based on their decomposition to carbon disulfide (CS_2) in an acid medium, followed mainly by spectrometry [6–10] and head space gas chromatography [11,12]. These methods are time-consuming, neither selective nor sensitive, and they do not let analysts distinguish between the subclasses of DTCs [1]. In addition, some published papers clearly demonstrate that CS_2 values determined by using the acid digestion method of crops rich in secondary metabolism of sulfur compounds have to be interpreted carefully [12]. However, up to now, these methods are used by authorities in Europe and USA to measure the presence of DTCs in different crops and legal limits have restricted only the amount of carbon disulfide [1–3].

An update methodology to determine DTCs and metabolites is indispensable to meet current analytical requirements [1]. Liquid chromatography (LC) and capillary electrophoresis (CE) with UV and/or electrochemical detection are the techniques most frequently used to discriminate and

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determine the different DTCs subclasses [13–24]. Determination of ETU and PTU in food commodities is achieved by LC with selective detectors or derivatization with a halogen or *S*-benzyl-*N*-(pentafluorobenzyl)-2-imidazolinethione and subsequent analysis by GC [3,11,25–28]. However, there is a clear trend toward the application of LC technique coupled with UV, electrochemical or MS detection [3,11,27,28]. For an unambiguous and simultaneous identification and quantification of DTCs and metabolites, LC in combination with MS, especially with the atmospheric pressure ionization (API) interfaces, offers new possibilities that have not been explored yet [1].

A still more difficult step is the sample preparation because the instability of DTCs, which are affected especially by pH, temperature and plant components [8,10,22,28]. The common techniques for the extraction and clean up of DTCs from matrices isolate these compounds by liquid extraction or solid-phase extraction (SPE) using, or not, some forms of ion-pairs [14,16,19,21–24]. ETU and PTU are polar and water soluble, and their extraction from fruits and vegetables is critical [3,11,27]. The prevalent approaches are based on liquid–liquid partitioning in dichloromethane or optional solid-phase cleanup on Extrelut columns, but they are very polemical since many authors encounter great difficulties to obtain acceptable and consistent recoveries [11,27]. In contrast, SPE on reversed-phase materials or matrix solid-phase dispersion (MSPD) methods have not so far been described.

Against this background, the objective of this paper was to develop and validate a simple, specific, and rapid method for the simultaneous determination of dazomet, disulfiram, thiram, ETU, and PTU residues in fruit and vegetables by LC–MS using SPE-based methods for the extraction and clean-up steps. For this purpose, atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) interfaces in positive ion (PI) mode were compared and optimized for the MS characterization of target compounds. Evaluation of several alternative approaches for the extraction and clean-up steps, SPE (using reverse materials) and MSPD, was also conducted to obtain reproducible recoveries. These procedures were used to analyze DTCs and metabolite residues in fruits and vegetables collected from different markets.

2. Experimental

2.1. Chemicals and reagents

Dazomet, thiram and ethylenthiourea were purchased from Riedel-de Haën (Seelze, Germany), disulfiram from Sigma (Steinheim, Germany) and propylenthiourea from Dr. Ehrenstorfer GmbH (Augsburg, Germany) with certified purity of at least 98%. Stock solutions of 1 mg ml^{-1} were prepared by weighing and dissolving each pesticide in methanol and stored at 4 °C. The solution of dazomet was stable under these storage conditions for almost 3 days and those of the other compounds for more than three months. Working solutions were daily prepared at different concentrations (0.25–50 μg ml^{-1}) by diluting aliquots of the stock solution in the same solvent.

HPLC-grade methanol, dichloromethane and acetone were purchased from Merck (Darmstadt, Germany). Deionized water (<8 MΩ cm resistivity) was obtained from MilliQ water purification system (Millipore, Bedford, MA, USA). Solvents were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain) before use.

Preparative C_{18} and C_8 sorbents were supplied by Análisis Vínicos (Tomelloso, Spain), Extrelut and Alumina by Merck, Florisil by Aldrich (Steinheim, Germany), silica gel by Scharlau and Supelclean ENVI-carb 120/400 by Supelco (Bellefonte, PA, USA).

2.2. Liquid chromatography with mass spectrometry

A Hewlet-Packard (Palo Alto, CA, USA) HP-1100 Series LC/MSD instrument with the HP Chemstation software version A.06.01 consisting of HP-1100 autosampler with a 100 μl loop, a G1312A binary pump, and a G1345A diode array UV-Vis detector coupled in series with a mass selective detector equipped with an atmospheric pressure ionization source usable as either APCI or ESI interfaces, was employed for the chromatographic analysis.

The operating parameters of the MS detector were: drying gas flow and temperature, nebulizer pressure, vaporizer temperature, capillary voltage, corona current, and fragmentor voltage, which were optimized by evaluating the sensitivity (signal-to-noise ratio) and fragmentation. For this purpose, each analyte, at a concentration of 10 μg ml^{-1}, was injected into the flow of the mobile phase using flow injection analyses (FIA) and detected in the full scan mode. The injection volume was set at 5 μl. The FIA was performed with an isocratic mobile phase for each analyte but the methanol percentage was varied from 10 to 90% depending of the compound in the way that this percentage was the same at which the analyte elutes from the analytical column with the gradient described in Table 1.

The LC separation was achieved on a 5 μm, 150 × 4.6 mm i.d. C_8 chromatographic column and security guard cartridge C_{18} (4 × 2 mm i.d.) both from Phenomenex (Cheshire, UK) using the gradient reported in Table 1.

Time-schedule selected-ion monitoring (SIM) of the most abundant ion of each compound was used for quantification. The optimum fragmentor voltages were pre-setted for each group of ions monitoring at the same time and automatically tuned using the instrument control utilities in the software. The optimal chromatographic and mass spectrometric conditions are listed in Table 1.

2.3. Sample preparation

Fruits and vegetables used for recovery trials were selected among groups of crops as indicated by the European

Table 1
LC–MS conditions

LC separation		
Solvent A	Water	
Solvent B	Methanol	
Flow rate (ml min^{-1})	0.8	
Time (min)	Methanol (%)	
Gradient elution		
0	10	
7.5	70	
12.5	90	
15	90	
	APCI	ESI
MS detection		
Drying gas flow (l min^{-1})	2.0	13.0
Drying gas temperture (°C)	250	350
Nebulizer pressure (bar)	40	60
Vaporizer temperature (°C)	250	–
Capillary voltage (V)	4000	4000
Corona current (μA)	4.0	–
Scan mode		
m/z range	50–350	50–350
s/cycle	0.42	0.42
Inter scan time (ms)	1	1
Fragmentor voltage		
Dazomet	50	60
Disulfiram	50	80
Ethylenethiourea	50	20
Propylenethiourea	50	20
Thiram	50	60
Selected ion		
Dazomet	163	163
Disulfiram	297	116/319
Ethylenethiourea	103	103/125
Propylenethiourea	117	117/139
Thiram	241	143/263

Union (EU) document on residue analysis [2]: (1) cereals and dry crops (rice and oat); (2) commodities with high water content (lettuce, cherry, peach and tomato); (3) commodities with high fat content (nut and avocado); and (4) fruits with high acid content (orange and lemon) to submit data for representative products. They were obtained from agricultural cooperatives and were also sampling in consonance with the guidelines of EU [2]; that is, as far as possible, the samples were taken at various places distributed throughout the lot (size ca. 50 kg). The sample weighed at least 1 kg and consisted of a least 10 individual fruits or vegetables.

The samples were prepared as is described in the Council directive 2002/63/EC [29]. Sample was cut in pieces and a representative portion of these pieces (200 g of fruit or vegetable taken randomly) was chopped and homogenized in a Bapitaurus food chopper (Taurus, Berlin, Germany).

2.3.1. Matrix solid-phase dispersion

A sample of 0.5 g was weighed and placed into a glass mortar (50 ml capacity) and gently blended with 0.5 g of the dispersing agent (see Table 5) for 5 min using a pestle, to obtain a homogeneous mixture. This mixture was introduced into a 100 × 9 mm i.d. glass column and conditioned with 0.2 ml of distilled water; then 20 ml of dichloromethane–methanol (80:20, v/v) were added to the column and the sample was allowed to elute dropwise by applying a slight vacuum. The eluent, which does not contain water, was collected in a graduated conical tube (20 ml) and concentrated, under a stream of nitrogen, to 0.5 ml.

2.3.2. Solid-phase extraction

A portion of 2 g of the sample was weighed and placed into an Erlenmeyer flask and homogenized with 25 ml of water by sonication over 15 min. The resulting suspension was filtered through Whatman 40 μm filter, and the filter cake was washed twice with 5 ml of deionized water.

The solution was passed under vacuum through a column containing 1 g of solid-phase (see Table 3) that was preconditioned with 10 ml of methanol and 10 ml of distilled water. The filtrate was discarded and the pesticides retained in the solid-phase were eluted with 10 ml dichloromethane–methanol (80:20, v/v). The eluent was collected in a graduated conical tube (20 ml) and concentrated, under a stream of nitrogen, to 2 ml.

2.4. Validation procedure

A test to verify the sample homogeneity was carried out because the relative small sample size requires a great degree of homogeneity to obtain representative samples. The intensive sample chopped must be performed carefully to minimize analyte degradation. The effect of homogenization on analytical accuracy was demonstrated on tomato spiked with a mixture of the studied DTCs at limit of quantitation (LOQ) level after selected the 200 g subsample prior to the homogenization step. After homogenization, this sample was analyzed seven times by the proposed carbon/MSPD method. The average recoveries found were between 40 and 75% and the relative standard deviations were between 10% for ETU and 20% for dazomet. These results and those obtained spiking directly the homogenized samples do not show apparent differences (see Table 6).

The following parameters were determined: linearity, repeatability, reproducibility, and LOQs, recoveries and matrix dependent variations as is established by the EU guidelines [2].

Linearity and matrix effects were evaluated by analyzing standard solutions and matrix matched standards at five points in the range of 0.25–50 μg ml^{-1} of each pesticide to cover the expected range of concentrations in samples.

LOQs were defined as the lowest level for which acceptable recoveries (70–110%) and repeatabilities (<20%) are obtained. However, it has been taken into account that when the recovery is not influenced by the analyte, concentration recoveries outside of this range are acceptable.

Intra- and inter-day precisions of the method were determined by the analysis of the five samples at each

concentration tested, in a day (intra) and in different days (inter). Recovery experiments were carried out in the selected plant materials spiked at the LOQ and ten times the LOQ levels of the studied compounds. For MSPD, 0.5 g of sample homogenized in a mortar was fortified with 500 μl of the stock pesticide solution. For SPE, 2 g of homogenized sample in an Erlenmeyer was fortified with 2 ml of the same solution. The flask was shaken manually to distribute the added standard as evenly as possible. The sample was left open at room temperature for 12 h to equilibrate the analytes in the vegetable. This practice was not detrimental for the analytes as it was demonstrated checking different equilibration times. The samples were analyzed according to the described methodologies.

3. Results and discussion

3.1. Liquid chromatography–mass spectrometry

Thiram, disulfiram, dazomet, ETU and PTU are weakly basic molecules, which produce easier positive ions than negative ones. Simultaneously, due to the ketol-thiol tautomerism, they exhibit acidic properties at pH values >10, which can favor the response in negative ionization (NI) mode. Preliminary experiments were performed to decide between both ionization modes. The studied analytes gave response in PI mode but not in NI mode at the concentrations studied.

Comparison of APCI and ESI interfaces was also carried out. Table 2 lists the main ions, their relative abundances and the limits of detection (LODs) obtained by both sources. Using an APCI interface, the only ion obtained for ETU, PTU and dazomet was the protonated molecule $[M + H]^+$ at *m*/*z* 103, 117 and 163, respectively. The mass spectrum of thiram showed a protonated molecule at *m*/*z* 241 along with fragment ion at *m*/*z* 120 corresponding to the loss of dimethylthiocarbamic acid. The mass spectrum of disulfiram gave a protonated molecule at *m*/*z* 297 and fragment ions at *m*/*z* 148 and 116 corresponding to diethylthiocarbamic acid and diethylthiocarbamoyl. According to the theory of APCI, a protonated molecule is generated in APCI. By fragmentation, neutral molecules are expelled, and the resulting fragments in most cases are protonated molecules again. To meet this criterion, the fragment molecules from thiram and disulfiram should be cyclic. A possible fragmentation pathway is illustrated in Fig. 1.

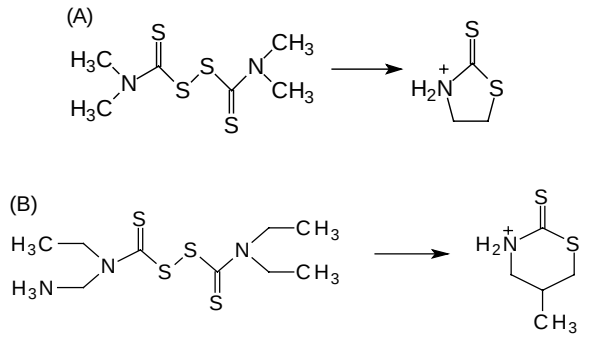


Fig. 1. Fragmentation pathways of the (A) thiram and (B) disulfiram.

The mass spectra obtained using ESI interface follow the same pattern reported for the APCI, but sodium adducts were always observed. This course is in agreement with the theory that the molecules need a functional group, which may donate a lone pair of electrons, to form stable sodium adducts [30]. DTCs show a great affinity for alkali metal ions and they gave exclusively sodium adducts, even in the absence of added sodium, excepting the fragment ion at *m*/*z* 116 (diethylthiocarbamoyl), which does not form adducts. ETU and PTU showed the protonated molecule $[M + H]^+$ and the sodium adduct $[M + Na]^+$ in the spectrum. Fortification of the sample with sodium ions prior to the injection affected the relative ion abundance but did not achieve a real improvement of the sensitivity.

Another relevant difference between APCI and ESI is the sensitivity. By APCI, the response varied from 1.25 ng for the most sensitive compounds, ETU and PTU, to 12.5 ng

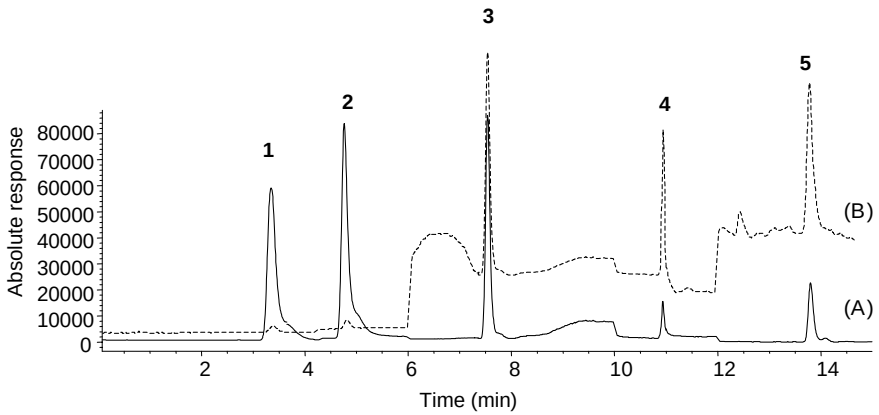
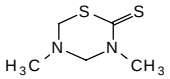
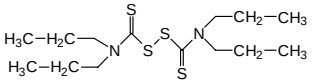
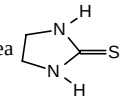
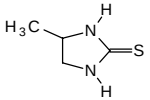
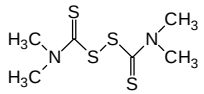


Fig. 2. LC–MS chromatograms in the SIM mode of a standard solution using (A) APCI and (B) ESI. Peak identification and concentrations: (1) ETU, 0.5 μg ml^{-1}; (2) PTU, 0.5 μg ml^{-1}; (3) dazomet, 1 μg ml^{-1}; (4) thiram, 1 μg ml^{-1}; and (5) disulfiram, 1 μg ml^{-1}.

Table 2
Comparison of APCI and ESI interfaces[a]

Pesticide	APCI		ESI	
	Main ions (relative abundance)	LOD (ng)[b]	Main ions (relative abundance)	LOD (ng)[b]
Dazomet 	163 (100) $[M + H]^+$	5	185 (100) $[M + Na]^+$	2.5
Disulfiram 	297 (100) $[M + H]^+$ 148 (17.7) $[M + H\text{-}HSCSN(CH_2CH_3)_2]^+$ 116 (30.2) $[M + H\text{-}HSSCSN(CH_2CH_3)_2]^+$	12.5	319 (99.3) $[M + Na]^+$ 171 (100) $[M + H\text{-}HSCSN(CH_2CH_3)_2 + Na]^+$ 116 (13.5) $[M + H\text{-}HSSCSN(CH_2CH_3)_2]^+$	2.5
Ethylenethiourea 	103 (100) $[M + H]^+$	1.25	103 (100) $[M + H]^+$ 125 (20) $[M + Na]^+$	5
Propylenethiourea 	117 (100) $[M + H]^+$	1.25	117 (57.7) $[M + H]^+$ 139 (100) $[M + Na]^+$	5
Thiram 	241 (100) $[M + H]^+$ 120 (26.7) $[M + H\text{-}HSCSN(CH_3)_2]^+$	12.5	263 (100) $[M + Na]^+$ 142 (74.9) $[M + H\text{-}HSCSN(CH_3)_2 + Na]^+$	5

[a] Calculated at the optimum fragmentor voltage (see Table 1).
[b] LOD, instrumental detection limits (ng injected); amount giving a peak with S/N = 3; determined in SIM mode by FIA.

for the least sensitive ones, thiram and disulfiram. While the ESI response was 5–10 times less sensitive for ETU and PTU than APCI, ESI was considerably more sensible for thiram and disulfiram than APCI. Fig. 2 illustrates the chromatographic response by both interfaces.

As it can be also observed in Fig. 2, the five analytes were well resolved using the methanol–water gradient reported in Section 2. Addition of modifiers with acid characteristics can affect the ion abundance and the sensitivity. Two aqueous solutions: 10 mM ammonium acetate (pH 4) and 10 mM ammonium formate (pH 3) were tested. The variations observed in the response were insignificant (<10%). The proposed conditions generate narrow and reproducible peaks and the separation of the five compounds was accomplished within 15 min.

Because the differences in sensitivity of APCI and ESI for the studied compounds a compromise should be achieved. APCI was selected because it achieves better sensitivity for ETU and PTU whereas the sensitivity for thiram and disulfiram is still enough for determining them at concentrations lower than the maximum residue levels (MRLs) established by the European legislation. In addition, it is more robust and gives reproducible spectra of the studied compounds without adduct formation.

Quality parameters were also determined. LC–APCI–MS provided a linear response over the range of injected amounts from 0.25 to 50 $\mu g\,ml^{-1}$ with good correlation coefficients (r between 0.981 and 0.997). For repeatability and reproducibility studies of the LC–MS procedure, five replicate determinations on the same day and on five different days of a standard solution (0.25 $\mu g\,ml^{-1}$ of ETU and PTU, 1 $\mu g\,ml^{-1}$ of dazomet and 2.50 $\mu g\,ml^{-1}$ of thiram and disulfiram) were carried out. Relative standard deviations (R.S.D.) ranged from 5 to 12% for run-to-run precision and from 8 to 16% for the day-to-day precision.

3.2. Optimization of different extraction procedures

The aim of this stage was to define the optimum conditions in terms of recovery for an extraction procedure, based on solid-phase cleanup of plant extract or direct MSPD. The analytes retained in the solid-phase or in the material dispersed were eluted with dichloromethane–methanol (80:20, v/v). This mixture provided the best eluting efficiency and removal of impurities (colorless solutions and cleaner chromatograms).

3.2.1. Solid-phase clean-up

The effect of pH, salt addition, solid-phase, organic solvent used to extract from plant materials and evaporation step was studied. The preliminary experiments to determine the influence of these parameters were carried out using pesticide solutions in pure water at concentration levels between 2.5 and 25 ppm.

Extrelut, C_{18}, C_8, carbon, silica, alumina and Florisil were checked as solid supports for SPE using a mixture of dichloromethane–methanol (80:20, v/v) as eluent. The results reported in Table 3 have shown that the best recoveries can be obtained by using carbon for all the compounds but the recoveries for ETU and PTU were very low (around 15%). The low recoveries obtained with adsorptive materials, such as alumina, Florisil and silica, are probably because these materials are deactivated by water as demonstrated the fact that the analytes can be found in the filtrate.

The addition of salt was also tested since ETU and PTU are highly polar and very soluble in water compounds. This effect was assessed by adding 10, 20 and 30% (w/v) of NaCl to water solutions containing the pesticides. The extraction efficiency of DTCs, ETU and PTU was not affected by the addition of salt. Samples were then extracted without salt addition to enhance the ruggedness of the method.

The studied compounds are stable over the pH range of 5–9. Therefore, the pH of pesticides solution was adjusted to pH 9 with 0.2% NaOH. The influence of the pH in the studied pesticides recovery was found to be insignificant.

The homogenization step from the plant material prior to SPE was also studied. DTCs were extracted from water, methanol–water (50:50, v/v) and water–acetone (50:50, v/v). The organic solvent was evaporated using a rotary evaporator prior to SPE. Results are reported in Table 4. Best recoveries were obtained homogenizing with water, probably because losses of DTCs, ETU and PTU occur during solvent evaporation. These results were confirmed repeating the analyses with peaches spiked with DTCs, ETU and PTU. The recoveries obtained from spiked aqueous solution were slightly higher than those obtained from spiked peaches as can be seen in Table 4.

Table 3
Recovery and relative standard deviations (R.S.D.) obtained by SPE from aqueous solutions using different solid-phases

Pesticides	Recovery, % (R.S.D., %, $n = 5$)							
	Concentration ($\mu g\,ml^{-1}$)	Alumina	Extrelut	Florisil	Silica	Carbon	C_8	C_{18}
Dazomet	1.00	3.2 (7)	–[a]	–	–	2.6 (10)	–	–
Disulfiram	2.50	4.2 (10)	–	29.3 (26)	29.3 (26)	89.1 (7)	99 (12)	82 (10)
Ethylenethiourea	0.25	–	–	–	–	13.7 (10)	–	–
Propylenethiourea	0.25	–	–	–	–	19.8 (12)	–	–
Thiram	2.50	2.4 (17)	–	11.5 (9)	4.6 (9)	80.7 (10)	108.2 (17)	99 (8)

[a] Indicates no recovery.

Table 4
Recovery obtained using different homogenization steps prior SPE from aqueous solutions and from spiked peaches homogenizing with water

Pesticides	Recovery, % (R.S.D., %, $n = 5$)					
	Concentration ($\mu g\,ml^{-1}$)	Acetone–water (50:50, v/v)	Methanol–water (50:50, v/v)	Water at pH 9 with 0.2% NaOH	Water	Peaches
Dazomet	10	51.1	53.2	60.5	55.1	53.7
Disulfiram	25	67.2	60.5	60.2	75.3	67.4
Ethylenethiourea	2.5	12.4	20.9	14.7	29.8	19.8
Propylenethiourea	2.5	8.8	26.4	22.4	11.4	8.7
Thiram	25	80.8	70.2	74.6	86.2	58.3

Different amounts: 2, 5, 10 and 15 g of peaches were tested. Between 2 and 10 g the recovery values were maintained. For 15 g the recovery were slightly lower. The sample amount used was 2 g in order to maintain the same amount of matrix per ml in the final extract using both extraction procedures. An advantage of increase the amount of sample is that the LOQs are improved.

3.2.2. Matrix solid-phase dispersion

The best dispersing agent to extract the studied compounds from plant materials by MSPD was checked. The preliminary experiments were also carried out with pesticide solutions in pure water at concentration levels between 1 and 50 ppm. C_8, C_{18} and carbon were tested. These results were confirmed using peaches spiked with the analytes. Recoveries shown in Table 5 display that the best results were obtained with carbon whereas C_8 and C_{18} failed to extract ETU and PTU.

3.2.3. SPE versus MSPD

Advantages of both methods are simple handling, low volumes of organic solvents or expensive reactives required, small sample amounts needed and high selectivity. The recoveries of both DTCs and metabolites are better using MSPD than SPE clean up. For MSPD using carbon, recoveries of DTCs and their metabolites were between 56.5 and 89.5% and 63.6 and 88.8% for standard solutions and for peaches, respectively. In SPE cleanup, these recoveries were between 2.6 and 89.1%, respectively. ETU and PTU are not retained in the solid-phase as a consequence of their high polarity and water solubility. The selection of MSPD as extraction procedure is obvious from data generated.

ETU and PTU recoveries were low using the SPE cleanup. However, this method could be, in some cases, an interesting alternative for the extraction of dazomet, disulfiram and thiram because the recoveries, precision and quantification limits obtained were very close to those obtained by MSPD. Taken into account that sensitivity is one of the most important parameters in pesticide residues determination, one feature of the SPE extraction cleanup is that the amount of matrix analyzed can be increased up to 10 g maintained the results, which can improve the LOQs more than five times.

3.3. Method validation

The proposed protocol, in which DTCs and metabolites were extracted by MSPD using ENVI-Carb and determined by LC–APCI–MS, was validated by the analysis of representative plant materials against a method based on dithiocarbamates decomposition to CS_2 and gas chromatography [11]. LOQs for the studied compounds ranged from 0.25 to 2.5 mg/kg, which are lower than the MRLs established by the Spanish and EU legislations [2,3] that are 3 ppm for thiram and between 1 and 5 ppm of CS_2 depending on the plant material. Table 6 shows the recovery and precision obtained at LOQ and 10 times the LOQ levels. In general, the recovery of the compounds was independent of their concentration in plant materials. Recoveries were from 33.2 (disulfiram at 10 × LOQ in lemon) to 109.0% (PTU at LOQ in lemon) and the relative standard deviations ranged from 4 to 21%.

Table 5
Recoveries and relative standard deviations (R.S.D.) by MSPD from standards and spiked peaches using different dispersing agents

Pesticides	Recovery, % (R.S.D., %, $n = 5$)						
	Concentration ($\mu g\,ml^{-1}$)	Standard solutions			Spiked peaches		
		C_8	C_{18}	Carbon	C_8	C_{18}	Carbon
Dazomet	1.00	81.7 (9)	61.8 (7)	89.5 (9)	20.1 (8))	19.4 (3)	77.9 (12)
Disulfiram	2.50	74 (13)	73 (12)	88.6 (3)	45.4 (6)	36.4 (8)	88.6 (7)
Ethylenethiourea	0.25	50.5 (9)	45.8 (10)	56.5 (15)	42.5 (5)	41.6 (10)	67.6 (10)
Propylenethiourea	0.25	43.2 (7)	45.9 (2)	58.9 (11)	50.6 (9)	54.5 (10)	63.6 (13)
Thiram	2.50	85 (8)	83 (8)	69.3 (10)	60.4 (7)	52.8 (7)	88.8 (4)

Table 6
Recoveries and R.S.D. obtained from different matrices spiked with the studied compounds at LOQ and 10 LOQ levels using carbon/MSPD method

	Recoveries, % (R.S.D., %, $n = 5$)									
	Dazomet		Disulfiram		Ethylenethiourea		Propylenethiourea		Thiram	
	0.5 mg kg^{-1}	5 mg kg^{-1}	2.5 mg kg^{-1}	25 mg kg^{-1}	0.25 mg kg^{-1}	2.5 mg kg^{-1}	0.25 mg kg^{-1}	2.5 mg kg^{-1}	2.5 mg kg^{-1}	25 mg kg^{-1}
Oat	44.0 (13)	45.2 (14)	78.3 (15)	73.2 (12)	40.2 (13)	45.3 (13)	44.2 (7)	45.7 (10)	70.4 (10)	73.7 (12)
Avocado	42.5 (16)	45.2 (10)	74.8 (14)	73.7 (11)	48.4 (4)	40.4 (8)	39.2 (17)	40.8 (10)	101 (9)	96.2 (10)
Cherry	68.9 (12)	70.2 (17)	76.9 (15)	80.7 (5)	49.4 (13)	45.6 (7)	40.6 (18)	49.4 (11)	84.4 (18)	85.6 (17)
Lemon	82.6 (16)	87.5 (16)	–	33.2 (7)	77.5 (13)	69.2 (14)	109.0 (16)	73.5 (12)	–	103.5 (18)
Lettuce	63.17 (7)	57.3 (4)	80.3 (16)	87.8 (8)	42.81 (20)	40.8 (5)	45.9 (11)	46.7 (10)	66.6 (19)	65.4 (11)
Nut	65.3 (13)	62.8 (17)	–	10.5 (19)	51.2 (14)	61.6 (15)	61.5 (14)	63.0 (6)	59.8 (11)	64.6 (14)
Orange	104.2 (10)	85.7 (18)	–	–	52.5 (10)	55.6 (21)	92.2 (19)	90.6 (13)	–	–
Tomato	41.5 (19)	40.5 (8)	71.5 (5)	70.8 (7)	42.8 (4)	47.6 (12)	52.18 (8)	50.1 (16)	71.6 (5)	74.8 (6)
Rice	49.0 (21)	42.3 (15)	83.1 (8)	87.4 (12)	52.9 (12)	50.7 (14)	53.1 (18)	54.2 (9)	84.34 (11)	85.3 (11)

The method is unsatisfactory in a certain number of pesticide/crop combination as fruits with high acid content (orange and lemon), for which thiram and disulfiram were not recovered, and nuts for which disulfiram was not recovered.

The hypothesis of thiram and disulfiram decomposition by the action of organic acids, juice of fruits and vegetables, or enzymatic reactions has been widely mentioned in the literature [8,15,21–25]. Heise et al. [8] postulated that procedures admitting small sample amounts and hence requiring an intense homogenization are not appropriated to determine these compounds because the contact between the residues on the peel of the fruit and vegetables and the flesh tissue

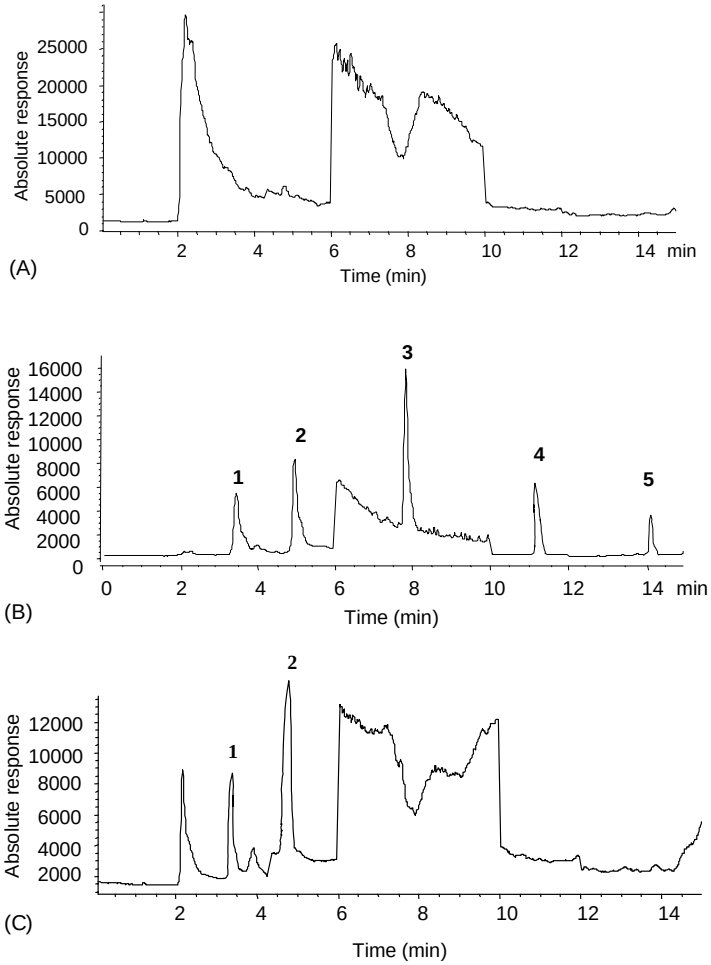


Fig. 3. LC–MS chromatograms in the SIM mode of (A) untreated control rice; (B) untreated control rice spiked at LOQ level; and (C) rice sample containing ETU at 0.32 mg kg^{-1} and PTU at 0.19 mg kg^{-1}. Peak identification as in Fig. 2.

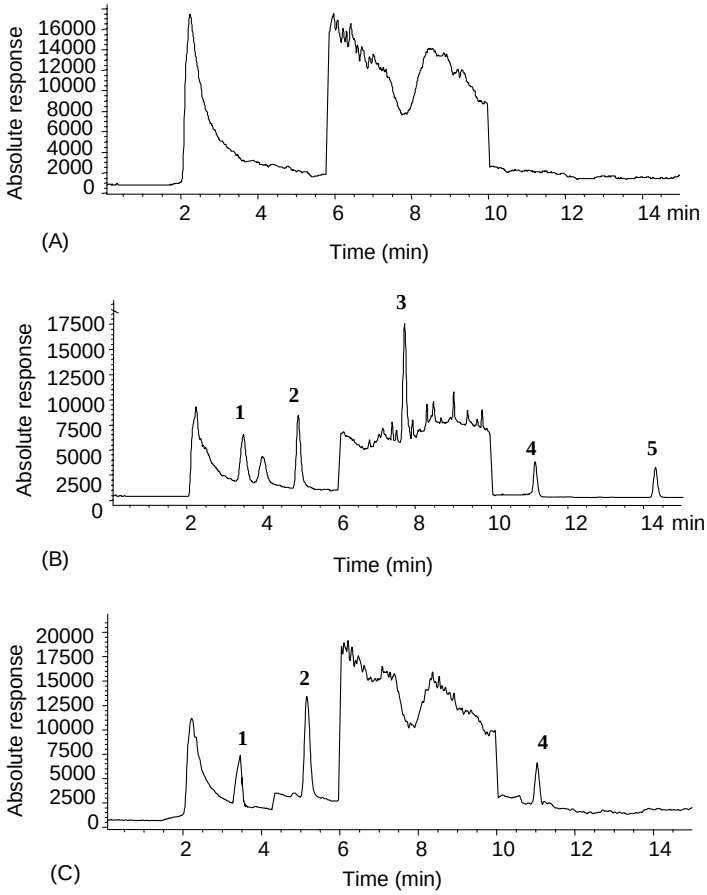


Fig. 4. LC–MS chromatograms in the SIM mode of (A) untreated control tomato; (B) untreated control tomato spiked at LOQ level; and (C) tomato sample containing ETU at 0.36 mg kg^{-1}, PTU at 0.54 mg kg^{-1}, and thiram at 2.9 mg kg^{-1}. Peak identification as in Fig. 2.

should be avoided. This argument favors the SPE cleanup previously tested because it would be able to extract pesticides residues located in the surface of the fruit sonicating the intact pieces with water, which could be more applicable for thiram and disulfiram extraction. On the contrary, ETU is readily absorbed and translocated from one part of the plant to another, requiring an important homogenization of the plant tissue [27,28,31]. It should be noted that thiram and disulfiram were only not recovered from fruits with high acid content; other plant materials tested showed recoveries of the same order than those reported for the extraction from dispersing material without matrix.

According to EU guidelines [2], the mean recoveries at each fortification level should be greater than 70%; in general, the values obtained using MSPD were around this value, except for ETU and PTU, which are recovered around 50%. Methods for determination of ETU and PTU have reported recoveries ranging only from 10 to 30% [3,11,27]. In this case, the proposed method is more efficient when compared to the cited ones.

A matrix interference study was carried out by comparing the area obtained for each compound in a standard solution with those obtained in a spiked blank extract of a commodity of each group (orange, tomato, rice and nut) at five concentration values from the LOQ to 50 mg kg^{-1}. Calibration graph of standard solutions and spiked blank extracts areas versus pesticide concentrations were constructed using a least-square linear regression. The presence of matrix always leads signal enhancement or suppression, which ranged from 0 to 20% depending on the matrix and on the compounds, but it has not shown any correlation between matrix type and enhancement and/or suppression. For consistent and accurate quantification, matrix matched standards were used in all analyses. The orange matrix matched standards gave not any problem with the thiram or disulfiram added.

The method was applied to the determination of fungicide residues in ten samples of each plant material taken from different markets of Valencia. ETU and PTU were detected in two samples of rice and thiram was found in one sample of tomato and one of peach. Examples of representative chromatograms for untreated control, spiked control at the LOQ levels and samples containing some of the studied compounds are given in Figs. 3 and 4.

4. Conclusions

The optimum results, in terms of sensitivity, selectivity and precision were obtained using ENVICarb for MSPD

followed by LC–APCI–MS. The proposed method provided determination of DTCs and metabolites in many representative plant materials, excepting disulfiram and thiram in fruits with high acid content and disulfiram in nuts. The main advantages are the small solvent and sample amount required, speed, low cost, cleaned blanks and the satisfactory recoveries, precision and LOQs provided. The DTC and metabolite residues determination in fruits and vegetables can be achieved with unequivocal identification of the compounds.

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