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A rapid and environmental friendly determination of the dithiocarbamate metabolites ethylenethiourea and propylenethiourea in fruit and vegetables by ultra high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Previous published methods for the analysis of ETU and PTU are time-consuming and furthermore use dichloromethane (DCM) for extraction or clean-up. This study details the development and validation of a rapid method that combines a simple extraction step with UHPLC–ESI$^+$-MS/MS. This is the first application of UHPLC–MS/MS to analyse these compounds. Besides that, we replaced DCM with a more environmental-friendly solvent. The analytical performance was evaluated with the analysis of spiked celery samples at 50 μg kg^{-1} (LOQ) and 300 μg kg^{-1}. The recoveries were between 65% and 90% for ETU and between 71% and 127% for PTU with RSDs in repeatability and reproducibility conditions below 10% for ETU. This method is rapid (a chromatographic run time of 2 min) and can easily be performed (no laborious clean-up). The presented method is environmental friendly with significant reduction in solvent consumption.

1. Introduction

Dithiocarbamates (DTCs) are one of the oldest and most widely used classes of organic fungicides in the world. DTCs have been used for crop protection for more than 40 years. Maneb, mancozeb, metiram, nabam and zineb are ethylenebisdithiocarbamates (EBDCs) and propineb is propylenebisdithiocarbamate (PBDC). These DTCs are widely used on different types of crops, including wine, top and stone fruit, vegetables, potatoes, tomatoes and nuts. They protect all these crops from various plant diseases [1,2]. They have low production cost and are polymeric complexes with transition metals, such as manganese in maneb or zinc in zineb, mancozeb and propineb [3,4]. These contact fungicides are not highly toxic but the toxicity is increased with the presence of heavy metal ion in the molecule. DTCs can cause eye, respirator and skin irritation when the exposure is short. With a long-term exposure, DTCs can cause dermatitis and skin sensitization [3]. DTCs are easily degraded in ethylenethiourea (ETU) or propylenethiourea (PTU) with the presence of moisture or oxygen and in biological systems. These degradation products do not show the same toxicity than their parent compounds. They are suspected to cause thyroid and neurotoxic effects [3,5]. ETU and PTU are suspected to induce carcinogenesis, teratogenesis and mutagenesis [1,5,6]. The dissipation of EBDCs was studied on different crops. These studies showed that no accumulation of ETU occurred but ETU is stable in water and is readily absorbed and metabolized by plants. Levels of ETU can increase when products are processed, especially during cooking. 16–23% (weigh basis) of EBDCs can be converted to ETU by heat treatment. Consequently, ETU concentrations may be higher in processed than in non processed foods [1,2,5,7].

For the determination of DTCs, several methods are described in literature [4,8,9]. Three methods are normalised by the European Committee for Standardization [9]. All these methods are based on the same principle. The sample is heated with hydrochloric acid and tin(II)chloride to release carbon disulfide (CS_2) from the present dithiocarbamates and/or thiram disulfide. These methods differ from their separation technique and detection method [9]. Unfortunately, only CS_2 is detected and consequently ETU cannot be measured. DTCs are determined by high performance liquid chromatography with an ultraviolet detector (HPLC–UV): dimethyldithiocarbamates (DMDs), EBDCs and PBDCs are identified based on their retention time [4,8,9].

With the intensively use of DTCs, a variety of methods have been developed for the analysis of their metabolites (ETU and PTU) in different commodities. These methods use high performance liquid chromatography with multiple detectors or gas chromatography with electron-capture and nitrogen-phosphorus detection [2,3,5,7,8,10–12]. Several sample preparation approaches are based

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on liquid–liquid extraction with dichloromethane with optional solid-phase (SPE) clean-up on Extrelut columns, or reversed-phase materials or matrix solid-phase dispersion (MSPD) [2,5,7,8,10–12].

An overview of analytical methods for pesticide residue analysis is presented by Pico et al. and by Soler et al. [13,14]. A comparison was made between the performance of different analytical techniques. The sensitivity of GC–MS(/MS) and LC–MS(/MS) were compared and the better performance of LC–MS(/MS) was concluded. Therefore, we chose LC–MS/MS to detect ETU and PTU.

As DTCs are already analysed by a method based on the standardised CEN method [9], our work focussed on the development and validation of an analytical method for the rapid identification and quantification of ETU and PTU in fruit and vegetables with ultra high performance liquid chromatography (UHPLC) coupled a triple quadrupole mass spectrometer (MS/MS) and positive electron spray ionization (ESI^+). These compounds are of importance for risk assessment with the respect to persistence and toxicology.

2. Materials and methods

2.1. Chemicals and reagents

Deuterated ethylenethiourea (ETU D4) and ethylenethiourea were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) with certified purity of 99%. Propylenethiourea Pestanal, maneb and propineb were obtained from Sigma–Aldrich (Seelze, Germany) with certified purity of 99%, 90.3% and 103.1% respectively. Distilled water (<8 MΩ cm resistivity) is obtained by the purification system (Millipore Milli-Q Water System, Bedford, USA). Methanol was of HPLC grade from Biosolve (Valkenswaard, The Netherlands) and trifluoroacetic acid (TFA) with certified purity of at least 99% (spectrophotometric grade) from Aldrich (Steinheim, Germany).

TFA was used as a solution of 0.1% in distilled water. The mixture of extraction was methanol–water (3/1).

The stock solution of internal standard, ETU D4, was prepared at 100 μg ml^{-1} in methanol and stored at −18 °C. Stock solutions of ETU and PTU were prepared at 1 mg ml^{-1} and stored in the same conditions. Dilute standards were prepared by dilution of the stock solution with distilled water and stocked at 4 °C. Solutions for calibration were prepared with blank extract of celery, melon or spinach, diluted standards and TFA 0.1%. These solutions are stocked at −18 °C.

2.2. Ultra performance liquid chromatography coupled with mass spectrometry

An Acquity UHPLC coupled with a Quattro Premier MS by Waters (Milford, USA) were used. This instrument consisting of a column manager, a sample manager with a loop of 5 μl and a binary solvent manager is equipped of MassLynx software version 4.1.

The LC separation was achieved on a 5 μm, 2.1 mm × 100 mm i.d. Uptisphere 5MM1 mixed-mode chromatographic column, with two different silanes (C8/SCX) bonded on silica, from Interchim (Montluçon, France) using isocratic conditions. Mobile phase containing 95% TFA at 0.1% and 5% of MeOH supplied at 0.45 ml min^{-1}. The injection volume was 5 μl in full loop to perform a better repeatability and run time was 2 min.

The mass spectrometer operating with electrospray ionization (ESI) in positive mode is used to acquire both the mass spectra (MS^1) and the product ion spectra (MS^2). It is programmed to allow the $[M+H]^+$ ion of ETU, PTU and ETU D4 to pass through the first quadrupole into the collision cell (Table 1).

Typical optimized ESI voltage settings were in Table 1. Nitrogen was used as the collision gas at a setting of 0.35 ml min^{-1}. The dwell time was 80 ms per channel for data collection. After 20 injections, the system was rinsed with MeOH during 2 min.

Table 1
MS detection and selected ion. Bold characters are used for quantifier daughter ions.

MS detection				
Capillary (kV)			0.3	
Cone (V)			31	
Extractor (V)			3	
RF Lens (V)			0.4	
Source temperature (°C)			130	
Desolvation temperature (°C)			450	
Cone gas flow (l/h)			51	
Desolvation gas flow (l/h)			800	
Selected ion	**Parents (*m/z*)**	**Daughter (*m/z*)**	**Collision (V)**	**Retention time (min)**
ETU	102.98	**44.3**	15	0.91
		85.9	15	
PTU	116.88	41.1	21	1.31
		58.0	13	
ETU D4	106.85	45.1	15	0.89
		48.18	15	

2.3. Sample preparation

The samples were cut into pieces and a representative portion of these pieces (500 g of fruit or vegetable taken randomly) was chopped and homogenized in Robot Coupe® R301 Ultra (Mont-Ste-Genevieve, Belgium). Prepared samples were stored at −18 °C until required for analysis.

2.4. Experimental set up for extraction

For extraction, 10 ± 0.1 g of matrix were weighed in a glass erlenmeyer of 100 ml with wide collar. We added 150 μl of solution of internal standard at 10 μg ml^{-1}. We added 20 ml of mixture of extraction and blended sample during 1 min with ultra-turrax homogenizer (Ultra-turrax IKA). The extract was filtered through büchner with Wathman paper no. 4, 42.5 mm. Ultra-turrax was rinsed twice with 2.5 ml of mixture of extraction. The solution was added on büchner. Filtrate was decanted in a graduated tube of 50 ml. A 500 μl aliquot of TFA 0.1% was added and mixture of extraction was used to give an extract volume of 30 ml. Extract is filtered on Mini-UniPrep PVDF Filter Media with Polypropylene Housing, 0.2 μm (Wathman, Florham Park, USA) before LC–MS/MS injection.

3. Results and discussion

Dithiocarbamates are widely used in agriculture as contact fungicide and can be decomposed into their metabolites ethylenethiourea and propylenethiourea. Due to their physicochemical properties, these metabolites have greater toxicity than the parent pesticides. For example ETU is suspected to have carcinogenic and teratogenic properties. Nevertheless, EU legislation has set maximum limits only for dithiocarbamates. Given the toxicity of the metabolites and its impact on public health, it is highly important to monitor these compounds. The present method has been developed within the framework of a study concerning the transformation of DTCs after processing.

3.1. Preliminary studies for method development

3.1.1. Extraction

Preliminary extraction tests showed out that the best recoveries were obtained with a mixture of MeOH and water (3/1).

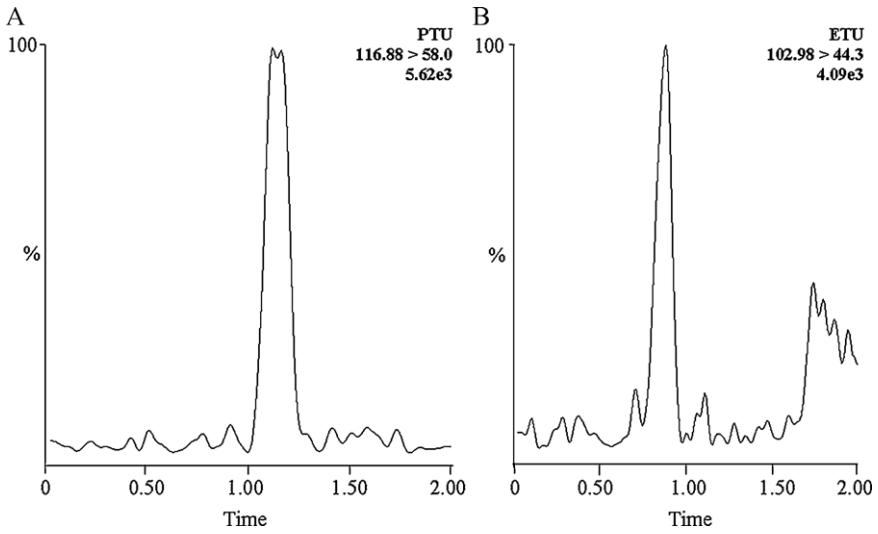

Fig. 1. Chromatogram of celery spike sample at 50 mg kg^{-1} for PTU (A) and ETU (B).

3.1.2. Chromatography

ETU and PTU are very polar molecules and are poorly retained on classical reversed phase column (C18). Blasco's et al. observed enough retention on a C8 column (150 mm × 4.6 mm, 5 μm) [8]. Tests on a C8 column for UHPLC (Waters® Acquity column 100 mm × 2.1 mm, 1.7 μm) showed that ETU was not retained. This phenomenon can be explained by the fact that there are less free silanol groups in an Acquity C8 than on a silica based C8 column. A large number of other columns with different brands were tested (all 10 cm length with a flow of 0.45 ml min^{-1}). HILIC (Waters) was tested with 95% ACN and 5% water. Even with only 5% of water, the retention time was 0.67 min. The best retention was obtained with Atlantis dC18 ($t_{R\,ETU}$ = 0.97 min; $t_{R\,PTU}$ = 1.55 min), Phenyl ($t_{R\,ETU}$ = 0.9 min; $t_{R\,PTU}$ = 1.36 min) and Uptisphere ($t_{R\,ETU}$ = 0.9 min; $t_{R\,PTU}$ = 1.12). In most cases, a huge matrix effect was observed. The absolute response of ETU and PTU was strongly reduced in the presence of matrix. Therefore, a purification step was introduced. Note that with Uptisphere column, the signal reduction observed was much lower.

3.1.3. Purification

Several proposed methods are laborious and pollutant, since they are using liquid–liquid extraction with DCM [2,5,7,8,10,12].

Solid phase extraction (SPE) is a much more rapid clean-up. Different types of SPE columns (C18, HLB, MCX) have been tested without success even using different conditions (solvent, pH, . . .). ETU and PTU was only retained Env+ from Isolute. The efficiency of the loading step is even better at pH bigger than 7. Unfortunately, recoveries for SPE felt down in the presence of matrix. Normal phase SPE columns (Cyanopropryl, Aminopropryl, Silica, Florisil) have been also tested for their retention capacities of interferences as chemical filters. The Cyanopropryl column removed satisfactorily some interferences. This implies a strong reduction of the matrix effect on Phenyl and Atlantis dC18 columns. In both cases, a good reduction of the matrix effect was observed. However, with Cyanopropryl/Phenyl of Cyanopropryl/Atlantis the suppression of the matrix effect was still lower than with Uptisphere column without SPE. On the Uptisphere column, the correction with the SPE clean-up was not significant.

In consequence, with two different silanes (C8/SCX) bonded on silica, Uptisphere column, compared with traditional phases offers a unique selectivity and was chosen. It minimized the interferences of the coeluting matrix and the second benefit of using this column is the elimination of a laborious purification step.

3.1.4. Ionization mode for MS/MS

Blasco et al. have compared two types of ionization mode, atmospheric pressure chemical ionization (APCI$^+$) and ESI$^+$ [8]. They obtained a better sensitivity with APCI$^+$. However during tests on our system, we demonstrated that the sensitivity was better for ESI$^+$ (test conditions are described Table 1).

3.2. Experimental set up

An environmental-friendly sample preparation strategy was chosen and DCM was replaced with MeOH and water. The analytes retained in the solid-phase were eluted with a mixture of MeOH–water. As explained in Section 3.1.1, this mixture gave the best extraction yield.

3.3. Validation study

The analytical method was in-house validated and the following method performance characteristics were obtained: detection and quantification limits, the linearity, the matrix effect, the recovery, the repeatability and reproducibility.

3.3.1. Detection and quantitation limits

The limit of detection (LOD), defined as the lowest concentration at which the analytical process can reliably differentiate from background levels, was accepted when the intensity of the signal is three times the background noise. The limit of quantification (LOQ) was defined when the signal to noise (S/N) is six [15].

The LOD–LOQ was calculated on a celery sample spiked at 25 μg kg^{-1}. Due to the decreasing signal in time (see Section 3.3.4), the LOD and LOQ were set at 25 and 50 μg kg^{-1}. Chromatogram of a celery spiked sample at LOQ is presented in Fig. 1.

3.3.2. Linearity

The linearity of the mass-spectrometric response was investigated by daily injecting standard solutions of ETU and PTU (50, 150, 300, 500 and 1000 μg kg^{-1}) during the validation.

Linearity was demonstrated by a correlation coefficient of 0.999 for ETU and 0.997 for PTU. The calibration lines are described by the equations $y = 0.0041x - 0.0214$ and $y = 0.089x - 0.1278$, respectively. In according to the SANCO document [16], all residuals observed are lower or equal to 10%.

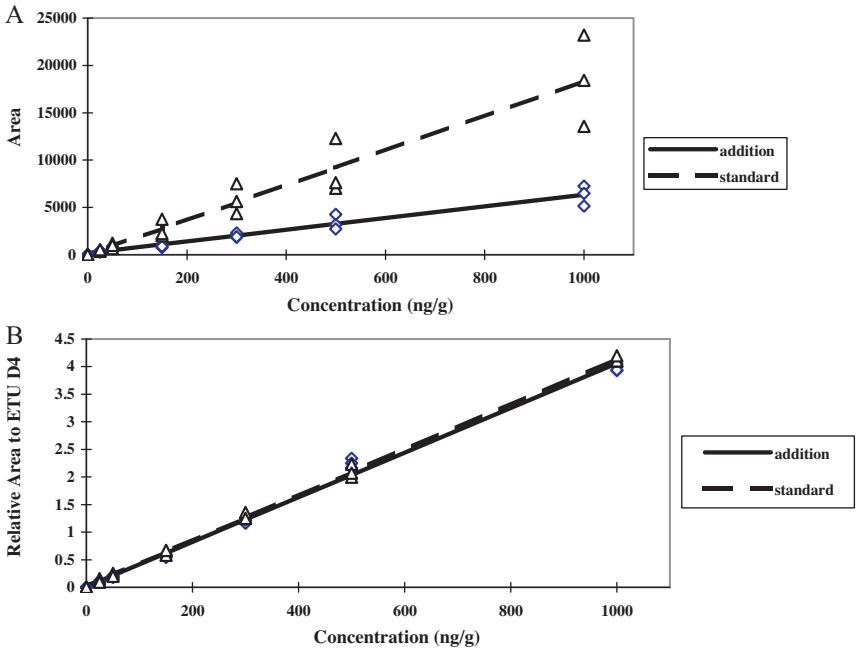

Fig. 2. Matrix effect without internal standard (A) and in presence of internal standard (B). The dashed line is ETU in solution and the other line is extract spiked with ETU.

3.3.3. Matrix effect

Matrix effect was evaluated by comparing calibration lines with standard solution and with celery samples matched standards at 5 identical concentrations levels.

As shown on the graph A in Fig. 2, a strong matrix effect for ETU was observed for celery sample. Preliminary tests had shown the same effect with melon samples and spinach samples. On the second graph (Fig. 2B), thanks to the addition of deuterated ETU, this matrix effect, which is of course always present, could be compensated. The results with internal standard were calculated with a rapport of the concentration of ETU and the concentration of deuterated ETU.

For PTU, a lower matrix effect was observed than for ETU, however the compensation with ETU D4 as internal standard was not satisfactory. Consequently, matrix matched standards must be used for quantification.

3.3.4. Precision and recovery

The precision and the recovery of the analytical method were evaluated on three matrixes samples (celery, melon and spinach) spiked at two concentration levels (50 $\mu g\,kg^{-1}$ and 300 $\mu g\,kg^{-1}$). 12 samples per concentration level and per matrix were analysed under repeatability and reproducibility conditions (4 analysis days and 2 operators). The precision is calculated according to the instructions in the SANCO document [12]. The recovery and precision results are resumed in Table 2.

The recoveries were between 65% and 90% for ETU and between 71% and 127% for PTU. Repeatability and reproducibility relative standard deviations (RSDs) were good, excepted for PTU. A decrease of signal was observed during one series of injections. A similar observation it was made by Startin et al. [12]. Consequently, an internal standard is absolutely necessary to minimize the signal variations in time and to have accurate results. No deuterated standard is available for PTU, which explains the lower results than for ETU.

For celery, RSD for repeatability are quite higher than for reproducibility, these results are a bit strange. But the long term reproducibility RSD for ETU is 9.2%. In general, results are better for celery and melon. This could be explained by the presence of more matrix interferences due to the greater coloration of the spinach extract. The recoveries and reproducibility RSDs of the method are equivalent or better than reported in literature [5,8].

3.3.5. Stability of dithiocarbamates during extraction process

The instability of DTCs, due to pH, temperature and matrix components, is the major challenge in sample preparation [8]. Consequently, to minimize the ETU and PTU formation the stability of maneb and propineb during the extraction process was evaluated. A solution containing 225 $mg\,kg^{-1}$ maneb and another containing 182 $mg\,kg^{-1}$ propineb was left on the bench from 30 min to 2 h before and after addition of the extraction solvent and after the complete extraction procedure was analysed. 0.4% of maneb was converted in ETU and 1.4% of propineb was converted in PTU. Consequently, 99.6% of maneb and 98.6% of propineb remained unchanged. The results show that the parent compounds are stable during the analytical procedure.

Table 2
Recoveries and precision determined for celery, melon and spinach at 50 $\mu g\,kg^{-1}$ and 300 $\mu g\,kg^{-1}$.

	ETU						PTU					
Matrix	Celeries		Melons		Spinach		Celeries		Melons		Spinach	
Spike level ($\mu g\,kg^{-1}$)	50	300	50	300	50	300	50	300	50	300	50	300
Recovery (%)	89.76	77.32	69.65	78.71	64.88	88.95	81.15	84.01	127.39	93.03	74.01	70.77
Repeatability (%) $n=6$	9.13	7.55	9.99	3.82	6.02	2.89	5.86	5.13	7.79	9.26	29.72	33.78
Reproducibility (%) $n=6$	8.28	7.61	11.79	7.25	14.02	10.12	12.76	21.14	14.36	27.29	21.27	36.22

4. Conclusion

An analytical method for quantifying ETU and PTU in fruit and vegetables has been developed. This method is rapid (a chromatographic run time of 2 min) and can easily be performed (no laborious clean-up). The presented method is environmental friendly with significant reduction in solvent consumption. In addition, the use of dichloromethane was avoided. It was demonstrated that a mixture of methanol–water is an excellent extraction solvent.

Without any clean-up of the extract, an important matrix effect was present with approximately 90% signal suppression when using standard C18 UHPLC columns. To overcome this setback, two approaches were explored. First, we did a systematic testing of different types of UHPLC columns to optimize the retention of ETU and PTU, which are highly polar compounds, while minimizing the interference of the coeluting matrix. The Uptisphere column offered the best results with a total chromatographic run-time of 2 min. The second approach was the use of an internal standard (deuterated ETU) to compensate for the important matrix effect. This technique was highly effective for ETU where no matrix effect was observed but in the case of PTU the calibration had to be done in matrix extract.

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