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Analytical Methods

Optimisation and validation of a specific analytical method for the determination of thiram residues in fruits and vegetables by LC–MS/MS

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ABSTRACT

Thiram is a non-systemic dithiocarbamate fungicide, which is easily degraded during sample preparation since it is affected by pH, matrix components and temperature. In this work, specific methodology for thiram analysis in vegetable (eggplant and lettuce) and fruit (strawberry) samples has been developed based on liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Minimising thiram degradation during standards storage and sample preparation was carefully studied. The effect of low temperature (about 5 °C), addition of a dehydrating agent (Na_2SO_4 anhydrous), pH regulator ($NaHCO_3$), and enzymatic activity reduction (EDTA) during extraction was evaluated. The optimised procedure was validated for eggplants, lettuces, and strawberries. Satisfactory recoveries, between 80% and 106%, and relative standard deviations below 10% were obtained at 0.1 and 0.01 mg/kg fortification levels ($n = 5$). Limits of detection below 0.0012 mg/kg were achieved. The validated method has been applied to eggplant and lettuce samples collected from different field trials as well as several strawberry and apple samples.

1. Introduction

Thiram (*Bis* (dimethylthiocarbamoyl)-disulphide) is a dithiocarbamate fungicide used as a seed protectant and in foliar treatments on fruits, vegetables, ornamentals and turf crops to control a number of fungal diseases, as well as to protect harvested crops from deterioration in storage or transport. It is also a degradation product of other dithiocarbamate fungicides as Ferbam and Ziram (Walia, Sharma, & Parmar, 2009). Thiram has been used in the treatment of human scabies, as a sunscreen and as a bactericide applied directly to the skin or incorporated into soap (Sharma, Aulakh, & Malik, 2003). In general, the fungical action of dialkyldithiocarbamates is a consequence of its capacity to inhibit metal dependant and sulphydryl enzymes (Blasco, Font, & Pico, 2004), such as pyruvate dehydrogenase (Roberts & Hutson, 1999). In addition, thiram is also used as an accelerator for rubber vulcanization and as rubber antioxidant (Szolar, 2007).

Regarding analytical methodology for dithiocarbamate residues in fruits and vegetables, problems related with their stability have been reported, especially in acidic plant juices (Heise, Weber, & Alder, 2000). Dithiocarbamates easily decompose into carbon disulphide (CS_2) and the respective amine in acidic medium (Crnogorac & Schwack, 2009). Because of this reason, it is not easy to achieve satisfactory recoveries for these compounds when a standard procedure is applied in multiresidue methods (plant sample homogenisation and solvent extraction) (Lehotay, de Kok, Hiemstra, & van Bodegraven, 2005; Paya et al., 2007). In consequence, determination of dithiocarbamates has been based for decades on their decomposition, using hot-acid digestion and collecting evolving CS_2 for its determination, and applying analytical techniques such as spectrophotometry or gas chromatography (GC) (Caldas, Conceicao, Miranda, de Souza, & Lima, 2001; Crnogorac & Schwack, 2009; Malik & Faubel, 1999; Sharma et al., 2003; Szolar, 2007; Vryzas, Papadakis, & Papadopoulou-Mourkidou, 2002). These indirect methods are time-consuming, have not sufficient sensitivity and do not allow distinguishing between different dithiocarbamates.

Regarding environmental samples, very little has been published on thiram residue analysis. Most analytical methods reported are based on liquid chromatography (LC) coupled to UV detector, not enough selective and sensitive. Sun and Lee (2002) developed a microwave-assisted extraction methodology for five carbamates, including thiram, PAHs, PCBs and triazines in soil samples. Since thiram was found to degrade seriously, it was no possible to calculate recoveries or limit of detection (LOD). An efficient SPE clean-up step to remove humic substances in aqueous samples for thiram determination by HPLC–UV has been described (Filipe, Vidal, Duarte, & Santos, 2007). The method was tested in soil extracts spiked at high levels, and LODs were around 0.33 mg/L.

According to Regulation (EC) No 396/2005 (Commission Regulation, 2005), all dithiocarbamates result in the final CS_2 residue;

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therefore, discrimination among them is generally not possible. Single residue methods should be implemented on a case by case basis when the specific quantification of thiram is required. In another document from European Food Safety Authority (EFSA) on Modification of the existing Maximum Residue Levels MRL for thiram in bananas (European Food Safety Authority, 2008), the following residue definitions for enforcement are established: for screening, total CS_2 residues; for cases of concern, thiram. When a specific and selective method is available, MRLs have to be expressed as thiram concentration. For selected matrices (eggplant, lettuce and strawberry) MRLs established are 0.1, 2, and 10 mg/kg, respectively, being 0.1 mg/kg the lower limit of analytical determination (Commission Regulation, 2005).

Consequently, to make available a specific MRL for thiram, it is necessary to develop reliable analytical methods for its individual determination at least at 0.1 mg/kg. LC coupled with tandem mass spectrometry (LC–MS/MS) is highly appropriate for polar/non-volatile analytes at low concentration due to its excellent selectivity and sensitivity, allowing the reliable identification and quantification of the analytes at low residue levels. Moreover, the acquisition of at least two specific transitions working in Selected Reaction Monitoring (SRM) mode leads to a reliable confirmation of the identity of the analyte detected in the sample (Pozo, Sancho, Ibanez, Hernandez, & Niessen, 2006). LC–MS/MS appears as an excellent technique that fits well with the physicochemical characteristics of thiram.

To our knowledge, only one analytical method based on LC–MS has been reported for thiram residue analysis. Blasco et al. have proposed a matrix solid-phase extraction followed by LC–APCI–MS determination for the simultaneous analysis of dithiocarbamates, including thiram, and their degradation products in plants (Blasco et al., 2004). Although the results were in general satisfactory, however thiram was not well recovered in some crops as fruits with high acid content (orange and lemon). Moreover, a limit of quantification (LOQ) as high as 2.5 mg/kg was reported for thiram in the rest of matrices, with recoveries between 60% and 101% in samples spiked at this concentration level.

Due to the lack of robust analytical methodology for thiram, the aim of this work is to develop an efficient and sensitive LC–MS/MS method for quantification and confirmation of thiram in fruit and vegetable samples. Special attention is given to relevant aspects like minimising thiram degradation during sample preparation and the stability of thiram in solvent, samples and raw extracts.

2. Material and methods

2.1. Reagents and chemicals

Thiram reference standard was purchased from Dr. Ehrenstorfer (Augsburg, Germany). HPLC-grade acetonitrile and methanol, residue analysis acetone and sodium sulphate anhydrous, reagent grade ammonium acetate and lithium acetate, reagent grade sodium hydrogen carbonate and synthesis grade ethylenediaminetetraacetic acid (EDTA) were purchased from ScharLab (Barcelona, Spain). LC-grade water was obtained by purifying demineralized water in a Milli-Q Gradient A10 (Millipore, Billerica, MA, USA).

Stock standard solution of thiram was prepared dissolving 50 mg powder, accurately weighted, in 100 mL acetone obtaining a final concentration of 500 μg/mL, which was stored at −20 °C. Working solutions, used for LC–MS/MS analysis or for sample fortification, were obtained by diluting stock solution with acetone and stored at −20 °C.

2.2. Instrumentation

An HPLC system Waters Alliance 2795 (Waters, Milford, MA, USA) was interfaced to a Quattro micro triple quadrupole mass spectrometer (Waters) using an orthogonal Z-spray-electrospray interface (ESI). The LC separation was performed by injecting 30 μL of sample in a Discovery C_{18} column (50×2.1 mm i.d., 5μm) (Supelco, Bellefonte, PA, USA), at a flow rate of 300 μL/min. The mobile phase used was a water–methanol gradient at 0.1 mM ammonium acetate where the percentage of methanol was changed linearly as follows: 0 min., 5%; 1 min., 90%; 3 min., 90%; 3.1 min., 5%. Sample vials in the auto-sampler of the LC system were at 5 °C. Drying gas as well as nebulising gas was nitrogen generated from pressurised air in a high purity nitrogen generator NM30LA 230Vac Gas Station from Peak Scientific (Inchinnan, Scotland). The desolvation and cone gas flows were selected to 600 L/h and 60 L/h, respectively. Infusion experiments were performed using the built-in syringe pump, directly connected to the interface.

For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Valencia, Spain) with a pressure of 3×10^{-3} mbar in the collision cell. Capillary voltages of 3.5 kV were used in positive ionisation mode. The interface temperature was set to 350 °C and the source temperature to 120 °C. Dwell times of 0.2 s were chosen for each transition. A solvent delay of 2 min was selected to give an additional clean-up using the built-in divert valve controlled by the Masslynx NT v.4.0 software. The quantification (Q) and confirmation (q_1, q_2, and q_3) transitions were $241 > 88$, $241 > 120$, $241 > 196$ and $241 > 76$, respectively, optimised at cone voltage of 15 V and collision energy of 10, 15, 5 and 35 eV, respectively. The application manager QuanLynx was used to process the quantitative data obtained from calibration standards and from crop samples.

2.3. Sample preparation

In order to avoid thiram degradation, sample chopping and extraction was carried out directly on frozen samples. All operations performed during sample preparation were done at low temperature (about 5 °C), with the material introduced in an ice bath. Frozen samples were quarterly cut into small pieces directly to obtain 50 g sample (precision 0.1 mg). Eggplant, lettuce, and apple samples were triturated and extracted, in presence of 50 g sodium sulphate anhydrous and 0.3 g EDTA, with 100 mL acetonitrile using a manual blender BP4576 vario mixer 750 (Ufesa, Barcelona, Spain). The entire extract was filtered through a 25–30 μm filter paper (Filtros ANOIA S.A., Barcelona, Spain), washed with 25 mL acetonitrile and the volume adjusted to 200 mL with acetonitrile. In order to remove small solid particles, an aliquot of the raw extract was passed through a 0.45 μm Nylon syringe filter (Scharlab, Barcelona, Spain). Finally, 30 μL of extract were directly injected in the LC–MS/MS system.

Sample extraction was slightly modified for acidic samples like strawberry. Double amount of Na_2SO_4 (100 g) was used, and 3.5 g of $NaHCO_3$ were also added during extraction following the same procedure described above, keeping the same amount of EDTA (0.3 g). The need to perform this modification will be lately discussed in the manuscript.

Matrix-matched standards were prepared using blank samples that were extracted accordingly to the sample preparation procedure. After extraction, an aliquot of the blank raw extract was transferred to a 10 mL volumetric flask, and 1 mL of the corresponding standard solution was added. Volume was adjusted to 10 mL with the blank raw extract.

Both, sample extracts and matrix-matched standards, were maintained all the time in an ice bath, at below 5 °C until analysis. If it was not performed within 8 h sample extracts and matrix-matched standards were put into the freezer at $\leqslant$20 °C. Moreover, sample vials were also maintained refrigerated at 5 ± 1 °C during LC–MS/MS.

Fortification of samples for recovery experiments was performed by delivering 1 mL of thiram standard solution (at 0.5 or