Analysis of Dithiocarbamate Fungicides in Vegetable Matrices Using HPLC-UV Followed by Atomic Absorption Spectrometry

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Abstract

A simple method combining ion-pair methylation, high-performance liquid chromatography (HPLC) analysis with detection at 272 nm and atomic absorption spectrometry was developed in order to determine 10 dithiocarbamate fungicides (Dazomet, Metam-Na, Ferbam, Ziram, Zineb, Maneb, Mancozeb, Metiram, Nabam and Propineb) and distinguish ethylenbisdithiocarbamates (EBDTCs) Zineb, Maneb and Mancozeb in diverse matrices. This method associates reverse phase analysis by HPLC analysis with detection at 272 nm, with atomic absorption spectrometry in order to distinguish, with the same extraction protocol, Maneb, Mancozeb and Zineb. The limits of detection (0.4, 0.8, 0.5, 1.25 and 1.97) and quantification (1.18, 2.5, 1.52, 4.2 and 6.52) calculated in injected nanogram, respectively, for Dazomet, Metam-Na, dimethyldithiocarbamates (DMDC), EBDTCs and propylenebisdithiocarbamates (PBDTCs) justify the sensitivity of the method used. The coefficients of determination R² were 0.9985, 0.9978, 0.9949, 0.988 and 0.9794, respectively, for Dazomet, Metam-Na, DMDC, EBDTCs and PBDTCs, and the recovery from fortified apple and leek samples was above 90%. Results obtained with the atomic absorption method in comparison with spectrophotometric analysis focus on the importance of the atomic absorption as a complementary specific method for the distinction between different EBDTC fungicides.

Introduction

Dithiocarbamate (DTC) fungicides are a group of organosulfur compounds represented by a general structure (R1R2) N-(C=S)-SX, where R can be substituted by an alkyl, alkylene, aryl or similar other group, and X is usually a metal ion, widely used in agriculture and frequently detected as pesticide residues in plant products in the world (1).

Their yearly consumption is between 25,000 and 35,000 metric tons (2, 3), distributed not only for crops protection but also for their role as accelerators for rubber vulcanization, as rubber antioxidants, as slimicides in tissue and paper as well as in sugar production, in waste water treatment, and as antifouling for water cooling systems.

DTC fungicides, used throughout the world, are divided into classes distinguished by their chemical structure and properties. These classes are ethylenbisdithiocarbamates (EBDTCs) such as Nabam, Mancozeb, Maneb, Metiram and Zineb; propylenebisdithiocarbamates (PBDTCs) such as Propineb; methylthiocarbamates such as Metam-sodium; dimethyldithiocarbamates (DMDC) such as Ferbam and Ziram and tetramethyiuram disulfide such as Thiram (4). The different structures of these different DTCs are summarized in Figure S1.

Among these compounds, Zineb is banned in the European Union because of the review on the entry in Annex I to Directive 91/414/EEC, in accordance to the Community Decision 2001/245/EC of 28 March 2001 (5). Nabam has not been included in the list of approved active
substances in the European Union. Then, no pesticide formulations are permitted with this agent in Germany, Austria and Switzerland (6).

However, the extensive use of these chemicals in agriculture has raised concern for their effects in occupational and ecotoxicological hazards. Usually, DTC complexes have low levels of toxicity, although some used pesticides have been found to produce adversarial health effects. The toxicological effects of DTC pesticides can occur from their absorption through skin contact, ingestion and inhalation. In fact, the lipophilic nature of DTCs makes them appropriate for their passage across the cell membrane (7). In addition, DTCs can lead to disturbances of the peripheral and the central nervous systems (8), as well as distal peripheral neuropathy induced by Mancozeb, Maneb, Metiram, Ziram and Thiram (9).

Thus, the persistence of these pesticides as residues in food can lead to serious problem causing chronic damage to health, as human consumes these substances as a part of their usual nutritional ingestion (10). For all these reasons, the detection of these fungicides in food crops has been a necessity. Gustafsson and Thompson (11) and Gustafsson and Fahlgren (12) established a method for determining EBDCs in food samples over their methylation to dimethylethylene-bisdiethiocarbamate (EBDC-dimethyl). This method is preferred for the analysis of EBDCs because the methyl derivative obtained has a characteristic structure associated with EBDCs. Contrariwise, this method does not allow distinguishing between the EBDCs differing only by the associated metal (13).

Most previous studies focused only on the analysis of a particular subgroup of DTCs, especially the EBDTCs and the PBDTCs (14, 15). In fact, several studies have been carried out to determine the persistence of these pesticides in food crops showing the importance of HPLC-DAD in such analysis (15, 16). The official technique used by authorities in Europe and the USA to determine the persistence of DTCs and their metabolites in crops is based on acidic digestion of the sample to transform DTCs to carbon disulfide (CS₂) and later quantification by either spectrophotometric absorption or gas chromatography (17, 18). However, these methods are time-consuming and do not differentiate between residues of individual class (18, 19).

In addition, further alternative analytical methods based on capillary electrophoresis, spectrophotometry or gas chromatography can be found in the recent literature (4). The use of liquid chromatography with mass spectrometry (MS) and tandem mass spectrometry (MS/MS) for the determination of EBDTCs has also been reported (13). In fact, all these methods were used for the extraction and identification of some of DTCs or of their metabolites but none of them describe a full validated method for all DTC fungicides.

On the other side, to the best of our knowledge, no previous studies focused on the analysis of all DTCs and the identification of different pesticides of a same group in a single simple extraction and analysis method. In addition, the method of Gustafsson and Thompson (11), returning to 1981 needs to be revalidated and updated over the years.

In addition, even though, the use of atomic absorption spectrometry complementarity with the high-performance liquid chromatography (HPLC) for the distinction between Zineb, Mancozeb and Maneb was reported by Lo et al. (20); authors studied a certain group of DTCs and did not develop and detail the protocol used which is important due to the effectiveness of the method.

Therefore, the aim of this study is to develop a method for the simultaneous analysis of 10 DTCs from different classes in one chromatographic run and apply it on various fruits and vegetable matrices, where the persistence of some DTCs could be extremely harmful. This method will also permit the simple differentiation of the type of EBDTCs in the same defined sample by the analysis of the water extract after methylation of the metal ion by atomic absorption spectrometry (FAAS). This latter technique will also be compared with UV–visible spectrophotometric method.

Experimental

Instrumentation

Analysis was performed by an HPLC equipped with an Autosampler 565 Kontron, a spectrophotometric diode array detector 545 V Kontron and two high-pressure pumps 422 S Kontron.

For the atomic spectrometry analysis, a Varian spectra 220 model FAAS equipped with deuterium lamp background correction, zinc and manganese hollow cathode lamps and an air-acetylene burner was used for the determination of zinc and manganese.

UV–visible spectrophotometric measurement was performed by using an UVIKON XL (Bio-Tek) Model UV–visible spectrophotometer with a 10-mm Quartz cell. The wavelength of maximum absorption used was 284 nm.

Chemicals and reagents

Acetonitrile, chloroform, n-hexane, 1–2 propanediol, methyl iodide, tetrabutylammonium hydrogen sulfate (55%) and methanol were purchased from Sigma-Aldrich (L’Isle D’Abeau, France), ethylene-diaminetetraacetic acid (EDTA) was obtained from Acros Organics, while sodium hydroxide (NaOH) and hydrochloric acid (37%) were purchased from Prolabo (VWR, France). Ultrapure deionized water was obtained from an Elga system.

Dazomet, Maneb, Mancozeb, Nham, Metiram, Proipineb, Thiram, Zineb and Ziram Pestanal® were obtained from Riel de Hain (Sigma-Aldrich, L’Isle D’Abeau, France), while Ferbam and Metam-sodium were purchased from Dr Ehrenstorfer GmbH.

Samples

Apples, leeks and tomatoes were purchased from local market in Strasbourg and pine needles were collected from the garden of the “Astronomical observatory” of the University of Strasbourg. This garden is situated in the main university campus close to the historical center of the town.

Solutions preparation

Alkaline EDTA solution (EDTA (0.25 M) in sodium hydroxide (0.45 M), with a pH –9.5) was obtained by solubilization of 7.3 g of EDTA and 1.8 g of NaOH in 100 mL of water.

Tetrabutylammonium hydrogen sulfate (0.41 M) and hydrochloric acid (2 M) were prepared by dilution with ultrapure water.

Methyl iodide (0.05 M) was prepared by dilution in chloroform–hexane (3:1) and 1, 2 propanediol (20%) was diluted in chloroform.

Methods

Extraction of DTCs from solution to monitor for extraction efficiency

Extraction of DTCs was performed according to the method of Gustafsson and Thompson (11) as follows: 1 mg of each DTC fungicide was dissolved separately with 5 mL of EDTA/NaOH by stirring for 5 min. Then, the extract was filtered and the extraction beaker and the filter were rinsed with 2 mL of water. The pH of the solution was adjusted to 7–7.5 by adding 1 mL of aqueous tetrabutylammonium
hydrogen sulfate solution (0.41 M) and 0.5 mL of HCl solution (2 M). The mixture was then transferred to a separatory funnel where 3 mL of methyl iodide in chloroform–hexane was added and then the mixture was extracted by shaking the separatory funnel. The organic phase was collected and the aqueous layer was re-extracted by 1 mL of methyl iodide in chloroform–hexane solution. Organic phases were combined and 0.5 mL of 1, 2 propanediol in chloroform (20%) was added. Extracts were allowed to evaporate and the residue was diluted in 5 mL of methanol. In total, 20 μL of the extract was injected into the HPLC system. Analysis was done in triplicate.

The aqueous layer was not discarded and was used for analysis by absorption atomic spectroscopy (FAAS).

### Analytical procedure

Separation was done on a reversed phase Macherey Nagel Nucleodur® C18 column (4.6 mm × 250 mm, 5 μm) at 1 mL min⁻¹ with a gradient of acetonitrile/water as follows: 35:65 for 15 min, 45:55 for 24 min, 35:65 for 5 min. Injection volume was 20 μL. The detection was done at 272 nm.

The instrumental FAAS parameters for zinc and manganese were as follows: wavelength of 213.9 and 279.5 nm, respectively, lamp current, 5 mA, bandpass, 0.5 nm, flow rate of 12 μL min⁻¹ for both metals.

### Analysis of DTC fungicides in fruit crops for method validation

In total, 10 g of each sample (leeks, apples) were cut in small pieces (~1 cm of dimension), fortified with 1 mg of each dithiocarbamate fungicides (Dazomet, Ferbam, Maneb, Mancozeb, Metam-sodium, Nabam, Propineb, Thiram, Zineb and Ziram), and analyzed immediately. The samples should not be homogenized, since homogenization shows a rapid breakdown of dithiocarbamates (21), the outer pieces of crops were analyzed. Samples were extracted with the same procedure as described above: first fortified samples were stirred for 5 min using 5 mL of EDTA/NaOH, then the extract was filtered and the extraction beaker and the filter were rinsed with 2 mL of water. The pH of the solution was adjusted to 7–7.5 by adding 1 mL of aqueous tetraethylammonium hydrogen sulfate solution (0.41 M) and 0.5 mL of HCl solution (2 M). The mixture was then transferred to a separatory funnel where it was extracted using 3 mL of methyl iodide in chloroform–hexane. The organic phase was collected and the aqueous layer was re-extracted by 1 mL of methyl iodide in chloroform–hexane solution. Finally, the organic phases were combined and 0.5 mL of 1, 2 propanediol in chloroform (20%) was added. Extracts were allowed to evaporate and the residue was diluted in 5 mL of methanol. In total, 20 μL of the extract were injected into the HPLC system (11, 12).

### Method application on pine needles

Pine needles (5 g) collected from Strasbourg have undergone the same extraction protocol cited above to examine their potential contamination by dithiocarbamates.

### Atomic absorption method to distinguish Zineb, Maneb and Mancozeb fungicides in food products

A well-known quantity of Mancozeb, Maneb and Zineb, as well as a mixture of the three fungicides, was added separately to tomato fractions, as a water suspension to prepare spiked samples (21). For preparing the spiked tomato samples, 2 mg of each pesticide were added to 10 mL of water separately and each suspension was mixed for 30 min with 10 g of tomatoes chopped into small pieces. Then, 10 mL of HCl (1 M) was added to the each spiked samples and the mixtures obtained were heated for 10 min in order to decompose completely the fungicides. The mixtures were then filtered with a filter paper to separate the food residue from the solution. The food residue was washed twice with 7 mL HCl (1 M) to offer the complete extraction of the fungicide. Filtrate and washings were collected together into a 100-μL volumetric flask and diluted with water. Then, zinc and manganese present in these solutions were determined by FAAS using a calibration graph drawn by using the metal (Zn and Mn) standard solutions. The FAAS determination procedure was repeated three times. Concentration of Zineb and Maneb was calculated using stoichiometry between metal and corresponding fungicides based on the molecular weight of the fungicide in comparison with the molecular weight of its metal (1 g of zinc is equivalent to 4.21 g of Zineb and 1 g of Mn is equivalent to 4.83 g of Maneb). In fact, the presence of Zn and Mn in the same extract indicates either the use of the two fungicides Zineb and Maneb or the use of Mancozeb (22).

Moreover, the aqueous layer obtained from the DTCs extraction in fruit crops was also analyzed with FAAS in order to verify the persistence of metals in this phase and then analyzing them to identify their original fungicide composition.

### Spectrophotometric method to distinguish Zineb, Maneb and Mancozeb fungicides

In order to check the accuracy and the selectivity of the atomic absorption spectrometry method for the distinction between EBDTCs fungicides, Maneb, Mancozeb and Zineb have also been determined by UV-Vis spectroscopy (21). Spiked tomato samples were prepared by adding separately 10 mL of each EBDTCs solution containing 2 mg of each pesticide to 10 g of tomatoes and mixing for 30 min. A mixture of all pesticides was also prepared. The spiked samples were then treated with 30 mL of NaOH (0.5 M) in order to dissolve the fungicides. The mixture was mixed with magnetic stirrer for 30 min to provide complete dissolution of the fungicides. Then, it was filtered with a filter paper and the residue was washed with two 10 mL portions of NaOH solution in order to provide the complete separation of EBDTCs. Filtrate was diluted to 100 mL with water; measurement procedure was repeated three times.

### Results

#### Method development

The analysis of each pesticide by HPLC coupled to UV-Visible detection shows that maximum absorbance was at 272 nm.

For the analysis of the mixture, 1 mg of each standard was solubilized together in the same beaker and extracted as cited above. Analysis was done in triplicate. Figure 1 shows the chromatogram of DTCs mixture showing the separation between subgroups of DTCs.

For the calibration curve, a dilution series was prepared from a stock solution of 50 mg L⁻¹, by dissolving 2.5 mg of each pesticide in 50 mL of EDTA/NaOH solution (Calibration range was done between 50 and 0.1 mg L⁻¹). In terms of quantities, the injected quantity for the calibration curve was between 2 ng and 1 μg of substance.
The retention time, the equation, the coefficient of regression, detection limit (DL) and quantification limit (QL) for each subgroup of DTCs are summarized in Table I.

Application to the analysis of dithiocarbamate fungicides in apples and leeks
To validate the method as well as to check its recovery, a known amount of each DTCS was added to various samples of apples and leeks. Samples were checked for their potential contamination in DTCs by analysis of each sample without spiking as blank.

Blank and spiked samples were extracted with the same protocol mentioned above and a complete absence of DTCs was verified for blank samples (Figures S2–S5). The results are given in Table II.

Analysis of Propineb fungicides in pine needles
The analysis of pine needles for their contamination of DTCs revealed the presence of a peak at 24 min corresponding to Propineb. In fact, the analysis was performed in triplicate and the concentration of Propineb found was calculated based on the corresponding calibration curve as follows:

\[
C = \frac{\mu g}{g} \text{ propineb} = \frac{A.Q_{\text{std}}}{A_{\text{std}} \cdot m} = 0.18 \mu g/g, \tag{1}
\]

where \(A\) is the peak area of Propineb in sample, \(A_{\text{std}}\) the peak area of Propineb standard, \(Q_{\text{std}}\) the Propineb quantity in considered peak (µg) and \(m\) the weight of sample in g.

Therefore, the amount of Propineb found corresponds to (0.18 × 5) = 0.9 µg of injected Propineb, which matches perfectly the calibration curve (injected quantities between 2 ng and 1 µg).

Atomic absorption method to distinguish Zineb, Maneb and Mancozeb fungicides
The general FAAS procedure mentioned was applied for the differentiation of EBTDCs in spiked tomatoes. Untreated tomatoes have also been analyzed and the correct Zinc and Manganese concentrations were calculated by the subtraction of the value obtained with untreated samples from the value obtained from spiked samples. Recovery results are shown in Table III.

The accuracy of the determination of Zineb and Maneb in spiked samples is reasonable, as relative standard deviation (RSD) in

both is 0.3 and the recovery is 95% for Zineb and 98% for Maneb which is also satisfactory.

Comparison of spectrophotometric method and FSAA to distinguish Maneb, Mancozeb and Zineb fungicides

The analysis of Maneb, Mancozeb and Zineb was also done spectrophotometrically in order to check maximum absorbance wavelength for these fungicides and to test the effectiveness of method for distinguishing EBDTC fungicides. UV–visible spectrum for the three pesticides as well as their mixture gave maximum absorption at 284 nm. Figure 2 shows the UV-Vis spectrum corresponding to the three EBDTCs mentioned.

Discussion

As expected by the reaction shown in Figure 3, EBDTC fungicides were eluted at the same retention time since their organic structures remain identical when the metal was removed. This is also the case of DMDTC fungicides.

Then, the only use of HPLC-UV/Vis method is not sufficient to differentiate fungicides of a same group as they are eluted at the same retention time. The use of atomic absorption spectrometry is consequently important for the quantification and identification of special dithiocarbamate pesticides like Maneb, Mancozeb and Zineb.

In addition, the method was validated on two different matrices, apples and leeks, in order to check its efficiency with matrix effects.

As shown in Table II, recoveries from leeks were more important than those obtained from apples and this may be due to the matrix effect of pectin present in apple leading to difficulties during filtration. However, no several matrix effects are present. In addition, the recoveries varied between different subgroups of DTCs and this may be due to interactions between different fungicides in the same subgroup. For example, recovery with Dazomet was 100%, whereas with EBDTCs, the interactions of different pesticides may be the cause of the decrease in the recovery rates.

Table II. Recovery of DTCs from Apples and Leeks

<table>
<thead>
<tr>
<th>Composition</th>
<th>Amount taken (mg)</th>
<th>Amount found (mg)</th>
<th>Recovery (%)</th>
<th>RSD %</th>
<th>Amount found (mg)</th>
<th>Recovery (%)</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dazomet</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>1.01</td>
<td>1</td>
<td>100</td>
<td>1.01</td>
</tr>
<tr>
<td>Metam-Na</td>
<td>1</td>
<td>0.95</td>
<td>95</td>
<td>1.61</td>
<td>0.98</td>
<td>98</td>
<td>1.02</td>
</tr>
<tr>
<td>DMDTCs</td>
<td>1</td>
<td>0.93</td>
<td>93</td>
<td>1.26</td>
<td>0.95</td>
<td>95</td>
<td>1.06</td>
</tr>
<tr>
<td>EBDTCs</td>
<td>1</td>
<td>0.92</td>
<td>92</td>
<td>1.10</td>
<td>0.94</td>
<td>94</td>
<td>1.65</td>
</tr>
<tr>
<td>PBDTCs</td>
<td>1</td>
<td>0.98</td>
<td>98</td>
<td>0.60</td>
<td>0.99</td>
<td>99</td>
<td>1.56</td>
</tr>
</tbody>
</table>

Table III. Recovery of Zineb and Maneb from Spiked Tomatoes

<table>
<thead>
<tr>
<th>Added Zineb concentration (mg/L)</th>
<th>Found Zineb concentration (mg/L)</th>
<th>Recovery (%)</th>
<th>RSD %</th>
<th>Added Maneb concentration (mg/L)</th>
<th>Found Maneb concentration (mg/L)</th>
<th>Recovery (%)</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.4 mg/L</td>
<td>8 mg/L</td>
<td>95%</td>
<td>0.3</td>
<td>9.6 mg/L</td>
<td>9.50 mg/L</td>
<td>98.9%</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Figure 2. UV–visible spectrum for Zineb, Maneb and Mancozeb.

Figure 3. Complexation reaction of DTCs.
These results are in accordance with those provided by Gustafsson and Thompson (11) and Gustafsson and Fahlgren (12), showing that this method is reliable not only for some of these DTCs but also for the totality of these fungicides. In fact, these results show a great separation between DTCs groups.

Furthermore, the SAA method and UV-visible spectrophotometric method were used as complementary method for the chromatographic analyses in order to separate EBDTC fungicides eluted at the same retention time.

In fact, the use of atomic absorption for differentiation between these two fungicides seems to be necessary, subsequent to the HPLC separation, as it allows differentiating EBDTCs based on the proportion of metals present in each DTC. The sample containing Zineb is rich in zinc and poor in manganese while the opposite is found for the sample containing Maneb. For samples fortified with Mancozeb, values cannot be anticipated as the ratio of zinc and manganese is different depending on the structure of Mancozeb. For that, the presence of zinc and manganese in the same sample can be only indicative of the use of this pesticide. In addition, the presence of Mn and Zn in the same extract could also mean a presence of Zineb and Maneb together, but as their effects are the same, as Mancozeb distinguishing between this mixture and Mancozeb seems non-significant.

Moreover, the results obtained by FAAS method emphasize the importance of this method provided by Turker and Sezer (22) and allows differentiating EBDTCs fungicide. This method can also be used to differ between DTCs class, e.g., EBDTC (Zineb) and DMDTC (Ferbam) as done by Turker and Sezer, but as we were only interested by the separation of EBDTCs, this method seems to be a method of choice.

Moreover, this technique is complementary to the chromatographic technique (HPLC-UV). The aqueous phases obtained from the extraction of apple and leeks enriched with DTC fungicides in the validation method give with the atomic absorption analysis high levels of zinc and manganese due to the presence of all DTC fungicides containing high levels of these two metals. These results show that from a single extraction, dithiocarbamates can be separated and fungicides belonging to a same subgroup can be differentiated by analyzing the aqueous phase containing chelated metals by EDTA.

In addition, the obtained results are consistent with those provided by Lo et al. (20) emphasizing once again the importance of the SAA as an efficient and complementary technique to the chromatographic analysis for a complete distinction of DTCs.

The complementarity between these two methods has several advantages: it reduces the error and the use of solvent and it is rapid, since no longer several extractions need to be done.

Contrariwise, the spectrophotometric method seems to be less important than the SAA. In fact, UV-visible spectrum obtained for the analysis of these three fungicides being very similar, then, this method may not be reliable for distinguishing between them, which emphasizes the importance of atomic absorption for this differentiation.

Results obtained with this method are not so significant as those supplied with Turker and Sezer (22), given that the goal was to differ pesticides of the same class having the same organic structure and differing only by metal, while Turker and Sezer (22) were interested by the distinction between two different classes (Ferbam and Zineb). For this reason, this method cannot be used as complementary to the present method.

Conclusion

The proposed analytical method developed in this study allows us to separate, to distinguish and to quantify residues of different subgroups of dithiocarbamates in a simple, efficient and responsive method. Moreover, the method was validated for natural samples and therefore it is well applicable in the food industry aimed at to know the persistence of these pesticides with potential toxicological effects. The application of the method to the native pine needles from Strasbourg shows the presence of Propineb constituting a direct application to the method. In addition, the distinction between different fungicides belonging to EBDTCs was realized by using flame atomic absorption spectrometry. In fact, the HPLC associated with atomic absorption provides a choice technique because of its efficiency and rapidity, once the pesticide is identified as EBDTCs fungicide by its retention time, then the atomic absorption can be applied based on the aqueous phase of the extraction. The advantage of the method is that no new preparations and extractions are required consequently minimizing uncertainties. Finally, the comparison of the atomic absorption technique to the spectrophotometric method UV-visible emphasizes the importance of the first technique as the results provided by the second were not so significant in terms of distinction between Maneb, Mancozeb and Zineb.

Supplementary data

Supplementary data are available at Journal of Chromatographic Science online.

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