

Investigation of the False Positive Results of Dithiocarbamate Pesticides due to Endogenous Nonpathogenic Carbon Disulfide in Organically Grown Plants (*Moringa oleifera*)

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Abstract: Moringa plant belongs to the family Moringaceae within the order Brassicales. It is a magical plant which contains 7 times the vitamin C content of oranges, 4 times the vitamin A content of carrots, 4 times the calcium content of milk, 3 times the potassium content of bananas and 2 times the protein content of yogurt (gram to gram). The most widely distributed species is *Moringa oleifera* Lam., referred as drumstick tree for the shape of its seed pods. Leaves of *M. oleifera* are rich in a unique glucosinolate named glucomoringin. Moringa were found to release CS₂ when submitted to experimental conditions of dithiocarbamate residue analysis. Gas chromatography was used to quantitate CS₂ and control samples were obtained from Moringa plantations cultivated in experimental areas, in which no treatment with fungicides of the dithiocarbamate group was applied. Endogenous CS₂ levels were compared with dithiocarbamate residues measured in Moringa samples from the field trials following applications of the macozeeb fungicide. Use small leaves resulted in an observed decrease in CS₂ concentration comparable to the large leaves. Temperature level used has an effect on the concentration of CS₂ measured as an indicator for dithiocarbamate pesticide residue. And use of high temperature (100 °C) in drying of Moringa leaves after harvesting may lead to some biochemical changes that caused an observed increase in CS₂ concentration comparable to drying at (50 °C). Evidence for formation of dithiocarbamate compound from moringin in moringa leaves are metabolized/detoxified principally by the mercapturic acid pathway. Conjugation with glutathione (GSH) promoted by glutathione transferases gives rise to the corresponding Glucosinolate - Isothiocyanate conjugates. These undergo further enzymatic modifications to give rise sequentially to the cysteinylglycine cysteine - Isothiocyanate and N-acetylcysteine-Isothiocyanate conjugates, all of which are dithiocarbamates. The detection of an ion with a m/z value 430.40, with the following fragments m/z 165.10 and m/z 121.05 respectively, among the ions analyzed by GC-MS gives a clear evidence for formation of dithiocarbamate by addition of glutathione to the isothiocyanate formed from glucosinolate in moringa plant.

Key words: Moringa, Gas chromatography, dithiocarbamate residues, endogenous, nonpathogenic carbon disulfide.

1. Introduction

This compound (glucomoringin) can be hydrolyzed by the enzyme myrosinase to its isothiocyanate counterpart, moringin. Most isothiocyanates are volatile viscous oils, unstable at room temperature and have high degradation rates [1]. Isothiocyanates from *Moringa oleifera*, are solid and have higher stability at room temperature. This might be attributed to the sugar moiety in their structure. Moringin is presumed

to be possible CS₂ precursor when *Moringa oleifera* leaves are tested for dithiocarbamate pesticide residue.

Isothiocyanate (e.g. moringin from *Moringa oleifera*) are electrophilic compounds and are subjected to conjugation with glutathione in aerobic organisms. This converts the isothiocyanate to dithiocarbamate [2]. The electron-deficient carbon atom of isothiocyanate is subjected to nucleophilic attack by glutathione. The addition of thiol group of glutathione to the electrophilic carbon of isothiocyanate forms dithiocarbamate. The isothiocyanates metabolism to dithiocarbamate is referred to as the mercapturic acid

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pathway [3]. At first, isothiocyanates are conjugated with glutathione (GSH) in a glutathione transferase (GST)-catalyzed reaction. It is followed by successive cleavage reactions catalyzed by C-glutamyltranspeptidase (GTP), cysteinylglycinase (CGASE), and N-acetyltransferase (NAT) to give isothiocyanate-N-acetyl cysteine.

Wolff et al. (2020) [4] suggested that *Moringa* isothiocyanate is protected from typical isothiocyanate metabolic modifications during uptake probably because of its unique glycosidic moiety. On the other hand, metabolites of the mercapturic acid pathway for

sulforaphane and iberin, the major isothiocyanates in broccoli, were detected and quantitatively measured in human plasma and urine after dietary consumption of broccoli [5]. One-pot production of dithiocarbamate from glucomoringin was reported [6]. Glucosinolates were hydrolyzed by the action of myrosinase in a phosphate buffer/dichloromethane biphasic system. This results in formation of dithiocarbamate (Fig. 1). Mechanistically this occurs by transformed moringin, the isothiocyanate intermediate, in the presence of a thiol, for example benzyl mercaptan, to trap the *in situ* produced moringin.

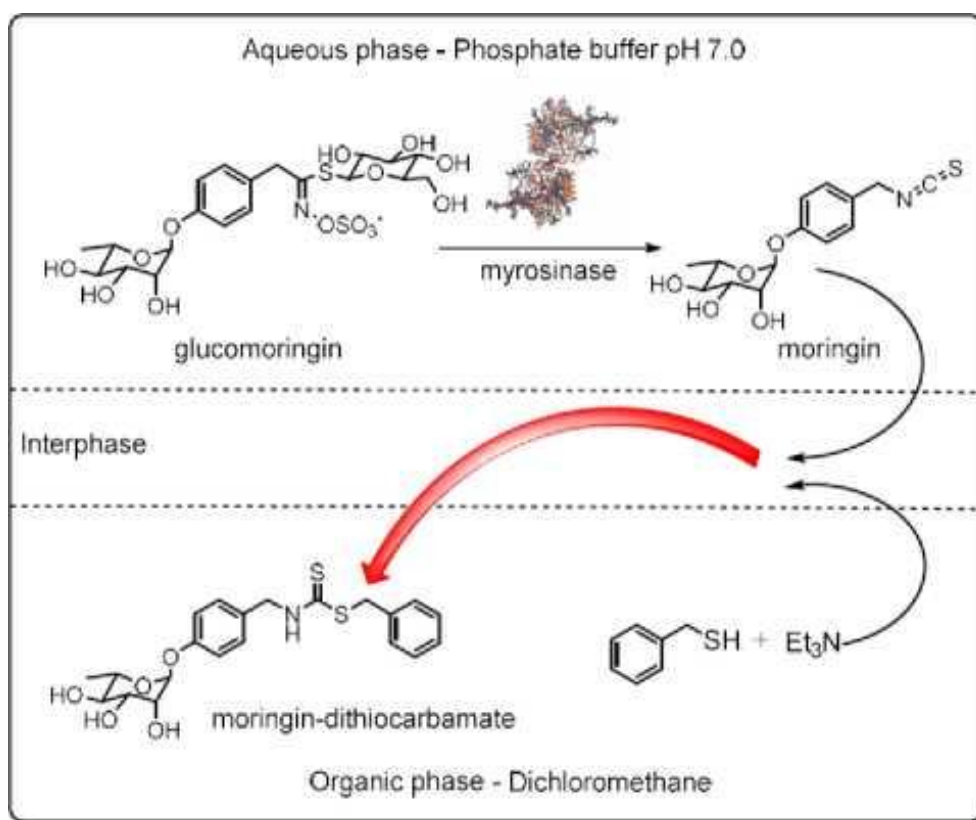


Fig. 1 One-pot production of moringin- dithiocarbamate from myrosinase catalyzed hydrolysis of glucosinolates in a biphasic system in the presence of triethylamine (NEt_3) and a model thiol. Aqueous phase is phosphate buffer pH 7.0 and organic phase is dichloromethane. Perz et al. (2000) [7] have reported the occurrence of a false positive result in the analysis of dithiocarbamate pesticide residue in members of the family Brassicaceae. This plant family is known for its content of various sulfur-containing compounds, such as mustard oil glycosides releasing isothiocyanates after enzymatic reaction or brassinines being antifungal indole derivatives.

2. Experimental

- GC-MS conditions consisted of Shimadzu gas chromatography--Model: TRACE --Thermo-Quest-part number:31709241 .-A HP-5 column (30 m x 0.25

mm, 0.25 μ m film thickness) was used and the GC temperature programed as follows: the column initial temperature was held at 60 $^{\circ}C$ for 2 min, followed by an increase to 220 $^{\circ}C$ at 5 $^{\circ}C/min$, and then increased to 280 at 15 $^{\circ}C/min$, after that held at 280 min for 10

min.- Helium was the carrier gas with the flow of 1.0 mL/min. The injection temperature is 250 °C and injection volume is 1.0 micro Liter in a split mode with a 1:10 ratio.

2.1 Identification of dithiocarbamate-generating compounds in *Moringa* leaves by Gas Chromatography

2.1.1 Isolation of moringin from moringa plant

The seeds of *Moringa oleifera* were used for extraction of the isothiocyanate moringin from moringa plant. According to Ribaud et al (2019) [8], the seeds contain the highest amount of the target compound. Moringin was isolated from the seeds using a published method [9].

- Moringa seeds were ground to a fine powder in a mortar to get seed meal.
- The seed meal was subsequently defatted with excess 77-hexane with frequent shaking for 24 h at 25 °C.

- The defatted seed meal was allowed to dry overnight.

- The defatted seed meal was mixed with 10 ml methylene chloride and 20 ml potassium phosphate buffer (0.05 M, pH 7.0). The mixture was kept at 25 °C for 8 h with frequent shaking to autolyze.

- Following this, 1 g sodium chloride and 1 g anhydrous sodium sulfate were added and mixed thoroughly.

- The methylene chloride layer was filtered and the residual paste was extracted three times with equal volumes of methylene chloride, which was combined and dried at 40 °C using rotary evaporator.

- The identity of the isolated compound was checked by measuring Nuclear Magnetic Resonance (NMR) spectrum. The assignment of each proton in the moringin molecule to its corresponding position in the structure was carried out according to published data [10]. The NMR spectrum is shown in Figure 2.

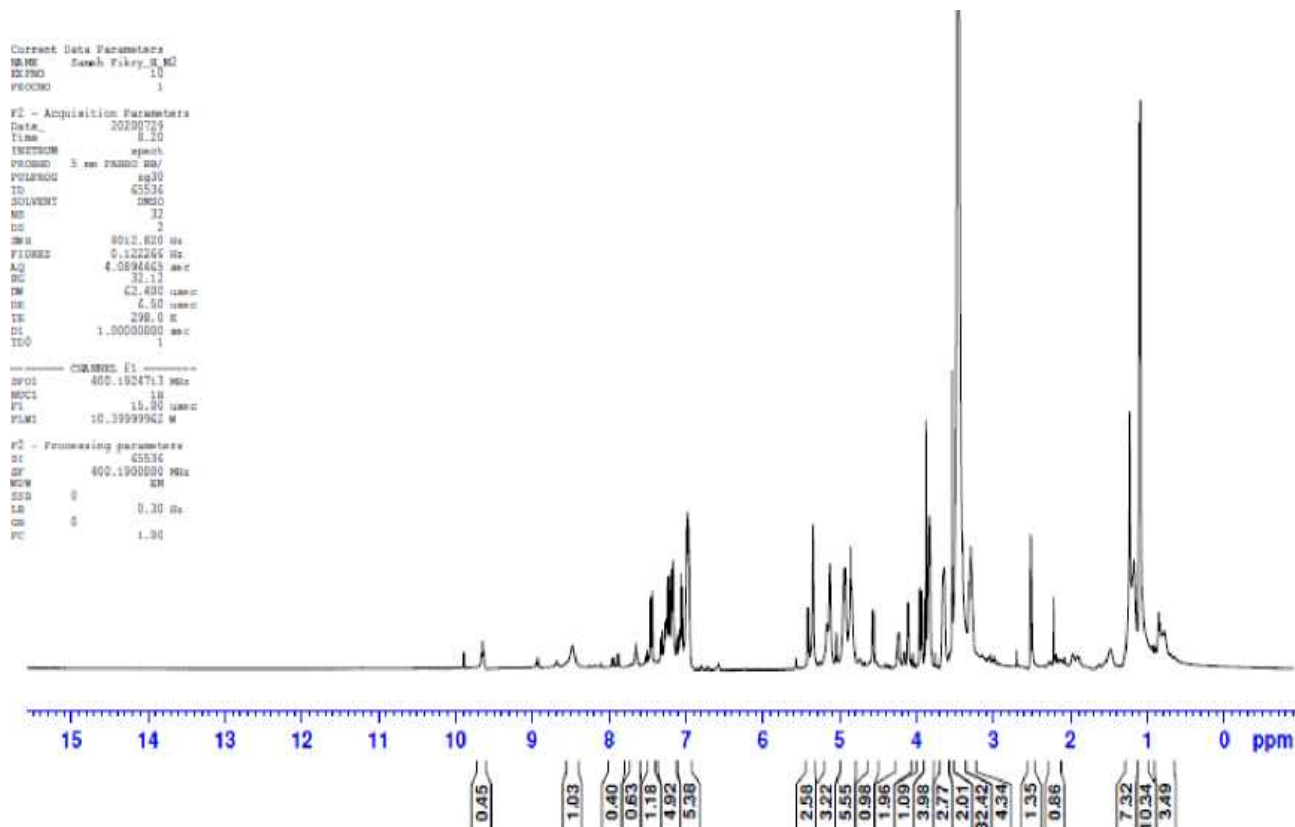


Fig. 2 NMR spectrum moringin isolated from the seeds of *Moringa oleifera*. The spectrum shows the signals related to moringin isothiocyanate glycoside with other impurities.

2.2 Effect of Concentration of the Pesticide "Mancozeb" on Carbon Disulphide Level Released

Aim: To establish the relation between the concentration of mancozeb pesticide and level of carbon disulphide released upon testing dithiocarbamate pesticide.

2.2.1 Analytical procedure

Mancozeb pesticide (Sigma) will be weighed (5, 10 and 20 mg) in the reaction bottles.

The different concentration level of the pesticide will be subjected to acid digestion using tin chloride/HCl dissolved in 500 ml concentrated hydrochloric acid (36%), the solution was decanted in one litre volumetric flask and complete to the mark with deionized water, use it as freshly prepared. Place 50 g sample and 40 ml isooctane was added and tin

chloride prepared, put it in water bath at 80 degree C for 60 minutes continuous mixing, cool and inject the upper phase using GC, Repeat the same process after spiking with carbon disulfide [11].

3. Discussion

The increase in mancozeb pesticide concentration will increase the level of carbon disulphide released upon testing dithiocarbamate pesticide using GC method

Determination of retention time of the solvent isooctane in blank using the temperature program, the solvent peak appears at retention time 8.89 min fig.3 and peak of carbon disulfide appears at 9.72 min fig.4 and both retention times are the same in the mixture of both carbon disulfide and isooctane fig.5.

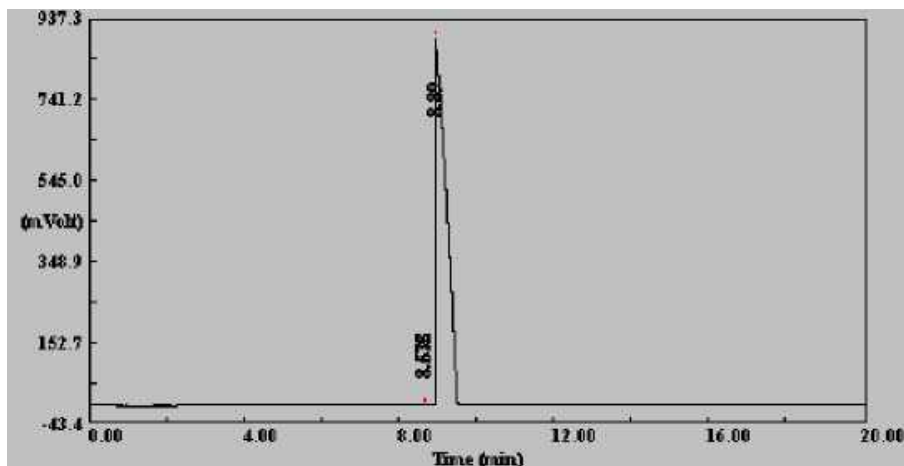


Fig. 3 R_t for the solvent isooctane = 8.89 min.

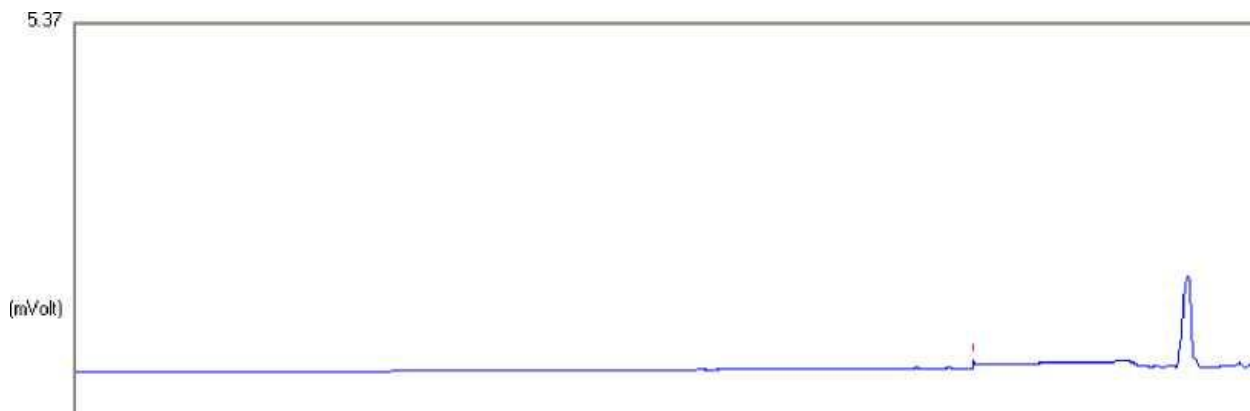


Fig. 4 Determination of the retention time of carbon disulphide (CS₂).

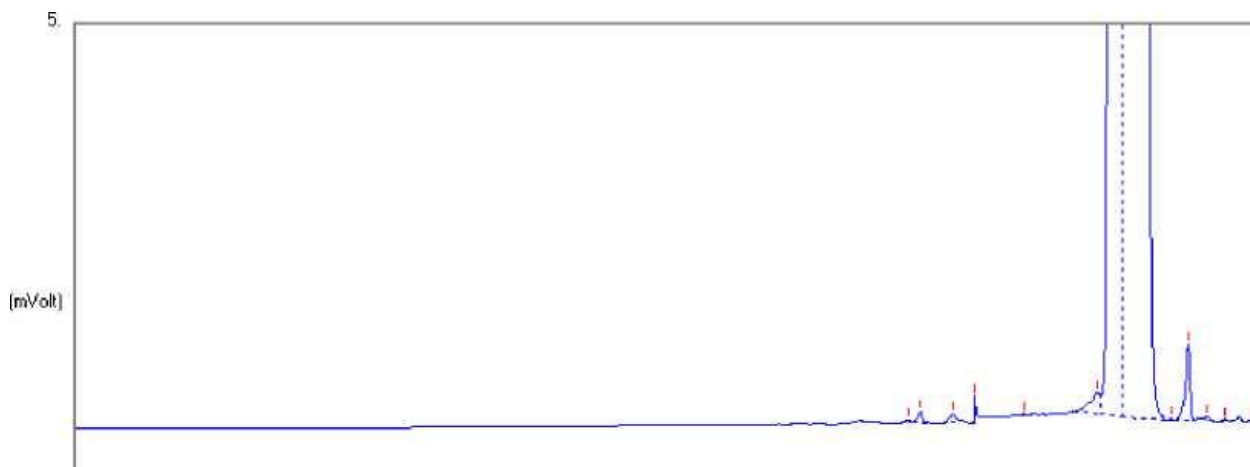


Fig. 5 Rt for the solvent isooctane = 8.89 min and Rt for the carbon sulphide (CS₂) = 9.72 min.

3.1 Effect of Age of Leaves on Carbon Disulphide Level Released upon Testing Dithiocarbamate Pesticide in *Moringa oleifera*

Aim: To establish the relation between the age of leaves and level of carbon disulphide released upon testing dithiocarbamate pesticide in the leaves of *Moringa oleifera*

Hypothesis: Harvesting mature leaves will result in increase the level of carbon disulphide released upon testing dithiocarbamate pesticide using GC-MS method. Small leaves contain lower level of moringin and will produce lower levels of carbon disulphide upon testing dithiocarbamate pesticide in *Moringa oleifera* leaves.

3.3.1 Analytical procedure

- *Moringa oleifera* leaves were collected from the plants cultivated at the Botanical Garden of Heliopolis University in the early morning. The leaves were separated into two categories small and large according to size of the leaf blade.

- The collected leaves (small and large separately) were dried at room temperature (ca. 30 °C) in shade for 3 days.

- The dried leaves (small and large separately) were grounded in a mortar to powdered form.

- The powdered leaves (small and large separately) were subjected to acid digestion using tin chloride/HCl - isooctane mixture at 80 °C for one hour

according to Cesnik and Gregorcic (2006) [11].

- Five microliters from the isooctane was injected into gas chromatography to estimate the produced carbon disulphide (CS₂).

4. Result

Small leaves will lead to different amount of carbon disulphide (CS₂), area (60) (Fig 6) while large leaves lead to ten folds the area, (580) (Fig 7). The amount of carbon disulphide (CS₂) should be the same if the origin of this compound is sprayed dithiocarbamate pesticide which will distributed equally on small and large leaves, but these results are logic in case that it is endogenously released product which increases as the plant grows.

Carbon disulfide in large leaves (ten folds the small ones at the same harvest and chromatographic conditions)

- High Resolution (HR) Liquid Chromatography - Mass Spectrometry (HR LC-MS)

Isothiocyanates are metabolized (detoxified) by the mercaptouric acid pathway through conjugation with glutathione (GSH) promoted by Glutathione transferases to give the corresponding GSH-Isothiocyanate conjugates. These undergo further enzymatic modifications to give rise sequentially to the cysteinyl glycine-, cysteine- N-acetyl cysteine-isothiocyanate conjugates all of which are dithiocarbamate.

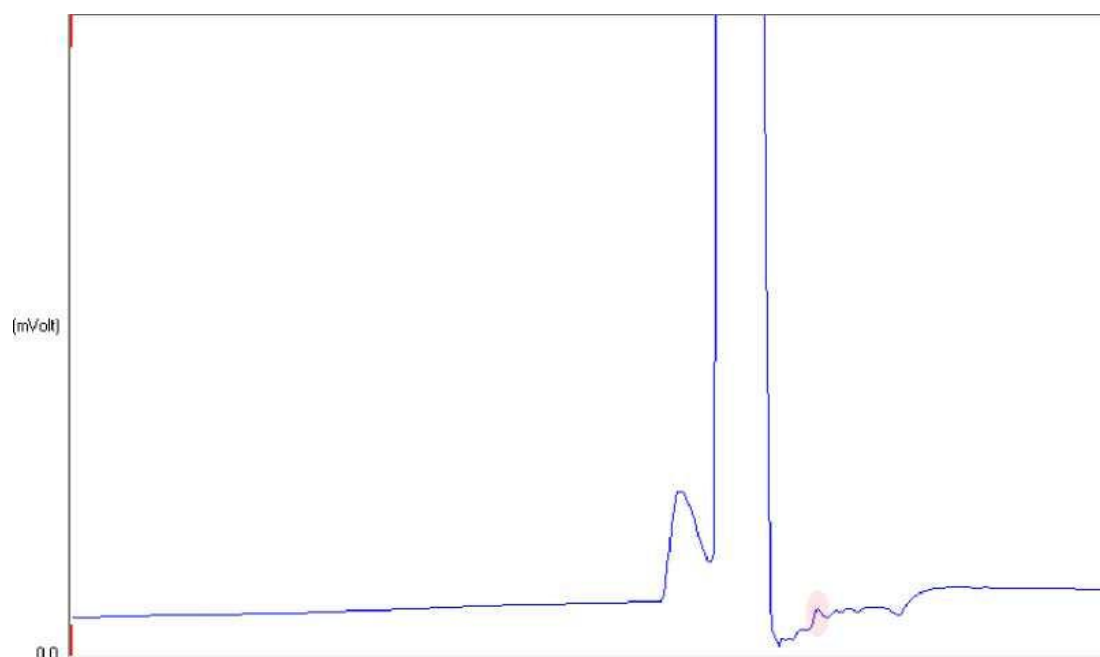


Fig. 6 Carbon disulfide in small leaves of moringa.

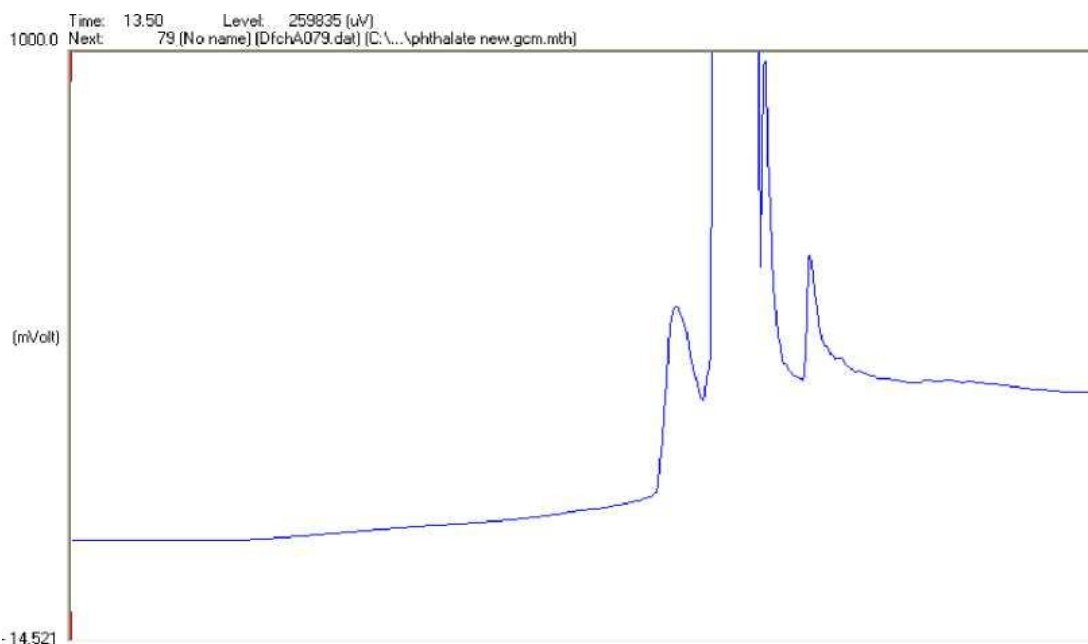


Fig. 7 Carbon disulfide in large leaves of moringa.

Detect an ion around m/z value 430 will give a clear evidence for formation of dithiocarbamate by addition of glutathione to the isothiocyanate High Resolution (HR) Liquid Chromatography - Mass Spectrometry (HR LC-MS) will be carried out to unambiguously identify the compounds in the Moringa leaves structurally related to the dithiocarbamate pesticide.

Unfortunately this technique (HR LC-MS) is not available in Egypt, so collaboration will be needed in this respect.

Experiment:

(1) Sonicate 1 g leaves of leaves in 50 ml methanol for 15 minutes twice, the methanol was evaporated by a rotary evaporator. Make three preparations.

Chromatographic conditions:

Column: C18 (250 mm* 4.6)

Mobile phase: Gradient elution with mobile phase A (0.1% TFA in distilled water), Mobile phase (0.1% in methanol) as follows 0 min: 10 min (100%A: 80%A), 10 min: 25 min (80%A: 50%A), 25 min: 40 min (50%A: 0%A)

Equilibration time: 10 min (100% A)

Detector wavelength: 280 nm.

4. Conclusions

The false positive result during testing of dithiocarbamate pesticide is due to endogenous isothiocyanate compound called moringin present in moringa leaves, this is evidenced by:

(1) Our GC-MS analysis (low resolution) is suggesting presence of a derivative from moringin compound containing the dithiocarbamate functional group.

(2) Analysis of dithiocarbamate pesticide content (as CS₂ mg/kg plant material) for the small and large leaves of *Moringa oleifera* plant separately showed 10 fold increase in the large leaves.

(3) Detect an ion around m/z value 430 will give a clear evidence for formation of dithiocarbamate by addition of glutathione to the isothiocyanate High Resolution (HR) Liquid Chromatography - Mass Spectrometry (HR LC-MS) will be carried out to unambiguously identify the compounds in the Moringa leaves structurally related to the dithiocarbamate pesticide.

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