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Characterization of the ultraviolet–visible photoproducts of thiophanate-methyl using high performance liquid chromatography coupled with high resolution tandem mass spectrometry—Detection in grapes and tomatoes

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ABSTRACT

UV–visible irradiation of thiophanate-methyl (TM) led to the formation of nine photoproducts that were characterized by high performance liquid chromatography coupled with high resolution Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS). Although carbendazime has been reported in the literature to be the major metabolite and photoproduct of thiophanate-methyl, it was not detected in this study. However, an isomer of carbendazime referred as PP2, which was unambiguously characterized owing to CID experiments, was found in great abundance. Grape berries and cherry tomatoes treated with aqueous solutions of thiophanate-methyl were submitted to irradiation under laboratory conditions. TM and PP2 were detected in both peel and flesh of berries. The ability of TM and PP2 to pass through the fruit skin has been shown to be highly compound and matrix dependent. *In vitro* bioassays on *Vibrio fischeri* bacteria showed that the global ecotoxicity of the TM solution increases significantly with the irradiation time. PP2 should likely contribute to this ecotoxicity enhancement since *in silico* estimations for *Daphnia magna* provide a LC50 value seven times lower for PP2 than for the parent molecule.

1. Introduction

Controlling crops pests that can lead to losses in the quality and quantity of farm yields is one of the major aims in modern agriculture. In this context, fungicides are molecules specifically used to prevent or limit the growth of fungal infections. During the preharvest period, these compounds are applied directly on plants and can be subjected to long-term sunlight exposure, leading to their photodegradation [1,2]. Simal Gandara and coworkers have paid particular attention to fungicides analysis in grape, wines and vineyard soils [3–8]. Thiophanate-methyl (dimethyl 4,4′-(*o*-phenylene) bis(3-thioallophanate)) is a commercial systemic fungicide belonging to the family of benzimidazole molecules. It was first introduced to the Japanese market by Nippon Soda Co., in 1970 [9]. This fungicide has a broad spectrum of action and can be used either for its protective or curative activity. Unlike several fungicides belonging to the same family (benomyl, ethyl thiophanate, carbendazim), thiophanate-methyl is still authorized in Europe. Its toxicity was extensively studied in the early 70s, concluding that no significant toxic effect was induced by the molecule [10–12]. However, Saquib et al. reported that thiophanate-methyl induced DNA damage in human lymphocytes [13]. Genotoxic and oxidative properties were also reported in 2014 by Ben Amara et al. in a study devoted to reactive oxygen species production in rat peripheral blood [14]. This molecule is also capable to bind to HSA (Human serum albumin) proteins, leading to molecular conformation changes such as a global decrease in alpha-helices forming these proteins [14,15]. Recent studies have also shown that thiophanate-methyl induces reprotoxic effects; cardone assessed this activity on *Podarcis sicula* and concluded that a long-term exposure to the fungicide leads to the degradation of seminiferous epithelium. An inhibition of the expression of steroid receptors was also observed and may cause infertility [16]. Another study on adult male rabbits revealed that thiophanate-methyl exposure led to a decrease in quantity and quality of sperm cells, a reduction in testosterone levels and histopathologic changes in epididymis [17]. The widespread and intensive use of this molecule for crop protection led to its detection, often above maximum residue limits, in several com-

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mercial samples such as rapeseeds, fruits, fruit juices and wines [18–23]. In this context, it must be kept in mind that thiophanate-methyl may undergo biotic and abiotic degradation leading to transformation products for which no toxicological data are available. LC–MS coupling is well known to constitute a technic of choice to determine fungicides in biological matrices [24] but HPLC coupled with sophisticated mass spectrometric methods associating high resolution and tandem mass spectrometry (HR-MS/MS) are often necessary to allow the characterization of metabolites and/or degradation products of emerging pollutants [25–27]. The photochemical behavior of thiophanate-methyl was studied in several matrices. Buchenauer et al. studied the photodegradation rate and mechanism of this molecule in aqueous solutions [28]. The authors reported that approximately 60% of the initial concentration was transformed in 2 days under outdoor environmental conditions, corresponding to a total sunshine duration of 22 h. The main identified photoproduct was methyl benzimidazol-2-yl carbamate (MBC), which is also known to be the main metabolite of thiophanate-methyl. On soil exposed to sunlight, the molecule showed S-oxidation at one of two C=S moieties and also the formation of MBC [29]. Soeda et al. showed that, on glass plates, thiophanate-methyl was rapidly phototransformed *via* the conversion of one C=S function into a ketone one and *via* intramolecular cyclization to from MBC. On grape leaves, apple leaves and bean plants, the major detected photoproduct after an exposition to sunlight was MBC [30,31]. The stability of this metabolite to photolysis was also studied in water and leaves. It was reported to be very stable with less than 10% lost after sunlight exposure for 40 h in aqueous solutions. On leaves of corn plants, no photolysis product were detected after sunlight exposition for 18 h [32].

In this context, the present paper aims to characterize the photoproducts formed during sunlight exposure in water and on fruits, to reassess the phototransformation mechanisms of thiophanate-methyl and to evaluate the impact of photolysis on its environmental toxicity. For that purpose, aqueous solutions were irradiated and analyzed by liquid chromatography coupled with mass spectrometry. The ability of the FT-ICR analyzer to provide ultra-high resolution mass spectra permits direct assignment of exact formulae while its capacity to perform tandem experiments may allow complete structural elucidation through differentiation of isomeric species. CID (Collision Induced Dissociation) experiments were performed on the ESI protonated photoproducts in order to establish their chemical structures. The ability of thiophanate-methyl and its main photoproducts to pass through the fruit peel was investigated on grape and cherry tomato samples. The toxicities of photoproducts were individually estimated *in silico*, with the Toxicity Estimation Software Tool (T.E.S.T.). Finally, the *in vitro* ecotoxicity of the irradiated solution was investigated, for several irradiation times, using *Vibrio fischeri* commercial test kits.

2. Experimental

2.1. Chemicals and reagents

Thiophanate-methyl (99.3% purity) and carbendazime (99.2% purity) were purchased from Sigma– Aldrich (St. Quentin Fallavier, France). Thiophanate-methyl and carbendazime (methyl benzimidazol-2-yl carbamate) will be referred as TM and MBC, respectively; their chemical structures are given in Fig. 1. Thiophanate-methyl-d_6 (hydrogen atoms of both methyl groups are replaced by deuterium atoms, 99.3% purity) was purchased from Cluzeau Info Labo (Courbevoie, France). Methylene chloride, acetonitrile and formic acid were purchased from Sigma Aldrich (chromatographic grade purity: 99.99%).

2.2. Sample preparation

Thiophanate-methyl aqueous solutions at 20 mg L^{-1} (solubility in water: 26.6 mg L^{-1} at 25 °C) were prepared in quartz tubes and sonicated for 10 min. A volume of 60 mL was prepared in each tube, in order to take ten 1 mL samples for kinetic studies without inducing an important change in the surface of solution irradiated (see Section 2.3). For the first part of this study devoted to photolysis in water, the aqueous solutions were irradiated as prepared. The collected samples (100 μL) were extracted by 100 μL methylene chloride for GC–MS analysis. For LC–MS analysis, samples were dried under a gentle nitrogen stream before addition of 100 μL of a H_2O/acetonitrile (90/10 v:v) mixture supplemented with 0.1% formic acid. For the second part of this work, dedicated to UV–visible irradiation on fruits, grape berries and cherry tomatoes were dipped in aqueous solutions of thiophanate-methyl at 5 mg L^{-1} (concentration normally used for crop treatment) for 5 min before being placed in quartz tubes for photolysis experiments. After irradiation, fruit peelings were carefully removed from the flesh using a lancet. Both Peel and pulp samples were separately weighted before being blended using a manual mortar. 1 mL water/acetonitrile 90/10 (v:v) mixture was added to each milling before centrifugation at 12 000 rpm for 90 min at 18 °C. Supernatants were filtered on Acrodisc® glass fiber syringe filters with polytetrafluoroethylene membranes (0.45 μm/13 mm) from Pall Corporation (New York, USA) before LC–HR-MS analysis.

2.3. Photolysis experiments

Photolysis experiments were mostly carried out on a laboratory-made reactor equipped with a high-pressure mercury lamp (HPL-N 125W/542 E27 SC; Phillips, Ivry-sur-Seine, France) delivering radiation at wavelengths ranging from 200 nm to 650 nm. According to manufacturer data, the incident radiation flux was 6200 lm. The reactor consists in six 120 mL quartz tubes disposed in a circle around the lamp and immerged into a sonicator (AL04-12, Advantage-Lab, Switzerland) filled with deionized water. During irradiation, the reactor is regularly cooled by water circulation to avoid uncontrolled heating of the irradiated solutions and to maintain a constant temperature of 40 ± 3 °C. For each experiment, 60 mL of a solution of thiophanate-methyl (see above) were used. To follow the kinetic evolution of photoproducts, a series of experiments was carried out with 10 irradiation times ranging from 0 to 120 min: 0, 10, 20, 30, 40, 50, 60, 80, 100 and 120 min. A constant pH value of 6.0 was measured for each irradiation time. All the irradiated solutions were analyzed by GC–MS and LC–MS. A reference solution of thiophanate-methyl was sonicated and kept 120 min at 40 °C without being submitted to irradiation. Analyses showed that thiophanate-methyl did not undergo any degradation under these conditions. In order to study the phototransformation of TM with a more realistic reproduction of full spectrum sunlight, analogous experiments were performed with a Q-sun test chamber (Xe-1-B/S, Q-Lab Saarbrücken, Germany) equipped with a xenon arc lamp (X-1800, Q-Lab, Saarbrücken, Germany) and a natural light filter (X-7640, Q-Lab, Saarbrücken, Germany). The lamp power was 1800 W and the irradiance 0.5 W/m^2. The photoproducts were the same than those obtained with the high pressure mercury lamp but with a thiophanate-methyl phototransformation rate about 20 times longer (irradiation times up to 40 h were used).

2.4. GC–MS operating conditions

In the present study, GC–MS analysis was conducted for the detection of photoproducts potentially insufficiently polar to be detected in LC–MC coupling. As a result, only the parent molecules and PP2 were detected in GC–MS which means that this method