GC-MS ANALYSIS OF *MALUS DOMESTICA* BARK AND LEAF EXTRACTS IN PESTICIDE NON-EXPOSED AND EXPOSED MATRICES

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GC-MS ANALYSIS OF *MALUS DOMESTICA* BARK AND LEAF EXTRACTS IN PESTICIDE NON-EXPOSED AND EXPOSED MATRICES (Abstract): **Aim:** Research on phytochemical constituents' existent in *Malus domestica* leaves and bark is a nowadays topic of interest especially due to the presence of various secondary metabolites with potential pharmacological characteristics. Quantifying the residual levels of singular pesticides, e.g. tebuconazole, and/or of chemicals in clearly stated combinational formulations is also of great interest especially due to all negative effects induced by pesticides on human health. **Material and methods:** The strategy within the present study corroborates field activities (i.e. clear treatment scheme, leaf and bark samples collection from the *Golden Delicious* and *Jonathan* apple varieties from an orchard in Miroslava, Iasi, Romania, before and after treatments spread) with sample analysis by gas chromatographic methods. Ultrasound-assisted techniques and clean-up procedures were used during the preparative steps. All methanol (MeOH) extracts were analyzed using a gas chromatograph, Agilent 7890A, in tandem with a mass spectrometer, Agilent 5975C, Agilent Technologies, United States of America. **Results:** Analysis of the chromatographic profiles obtained for the investigated pesticide non-exposed matrices revealed the existence of many potential therapeutic bioactive compounds with positive benefits on human health (e.g., lupeol, friedelin, α-tocopherol known also as vitamin E, mellein). Chromatographic run profiles of methanol extracts associated to sample matrices collected at different periods within the treatment scheme revealed the existence of the chromatographic peak corresponding to tebuconazole at a retention time of 13.75 minutes. Pesticides existent in the in-use clearly stated combinational formulations have been as well identified. **Conclusions:** A preliminary characterization of the secondary metabolites existent in *Golden Delicious* and *Jonathan* apple leaves and bark was achieved for the first time for samples collected from an orchard in Miroslava, Iasi, Northeastern Romania. In pesticide non-exposed matrix compounds of pharmacological interest have been identified. Clear evidence has been obtained about the existence of tebuconazole and of other pesticides from the in-use clearly stated combinational formulations, in all samples collected in a well-established time span from the treatment spread. Samples containing residual pesticides might induce possible negative consequences for human health. **Keywords:** TEBUCONAZOLE, *JONATHAN*, *GOLDEN DELICIOUS*, LEAF, BARK.

In temperate geographic areas apple tree is one of the most widely cultivated fruit trees (1). Apples, as rich sources of phytochemicals, are the fruits extensively used worldwide (2).

Minerals such as nitrogen, phosphorus, potassium, calcium, magnesium, iron, copper, manganese, zinc are present in important amounts in apple leaves (3). Apple leaves and bark are often assigned as potential sources of antioxidant compounds (4,5,6). Flavonoids and other phenols from plants, such as phenolic acids, stilbene, tannins, lignans and lignin, are particularly common in leaves, flowers and woody tissues, such as stems and bark (7).

Nowadays, tebuconazole (TEB) which is a systemic triazole fungicide is intensively used to combat foliar diseases in fruit trees (8). This triazole fungicide group inhibits lanosterol 14∝-demethylase (CYP51) activity by disrupting pathogen's membrane and by inhibiting pest's cells growth. It has been already shown that tebuconazole is highly efficient against pathogenic fungi from various plants and that it is widely used for controlling white rot disease. Important quantities of apple are exported from China (9) to Europe (10), and studies performed in field on Chinese crops have shown that tebuconazole is an excellent fungicide for controlling white apple rot (9). There are studies suggesting that tebuconazole is highly efficient in combating mold and rust in fruit, and that once these tebuconazole-containing fruits are consumed they can exert serious health consequences (11).

MATERIAL AND METHODS

Treatment and sampling. Clearly stated combinational pesticide formulations, containing mainly tebuconazole, were used during sample treatments. The interest products have been sequential sprayed (4 times) in full agreement with the producer's recommendation (Bayer) and the samples (leaves, bark) have been collected by following an own developed strategy in the April 9, 2016 – October 6, 2016. No significant precipitation events have been recorded during treatments and sampling steps, Iasi region facing mainly drought throughout the period of interest.

Sample preparation. Samples collected from two varieties of apple, i.e. *Golden Delicious* and *Jonathan*, were subjected to several preparative steps for further analysis by GC-MS technique. Accurately weighted amounts from each matrix of interest, whether or not previously subjected to field treatment with tebuconazole and clearly stated combinational pesticide formulations, each in normal and double dose, were passed through ultrasound assisted extraction process in two different solvents.

To increase extraction efficiency and the requested accuracy for a specific study on pesticide dynamics in the field, each sampled matrix was well shredded with a scalpel. This step was immediately followed by mass weighing on an analytical balance Adam PW254 with an accuracy of 10^{-4} g. For each matrix ~ 0.2 g of sample was weighed (i.e. *Golden Delicious* and *Jonathan* matrices, two modes of treatment, singular tebuconazole and with clearly stated combinational pesticide formulations, each at normal dose and double dose, at each collection point).

Each weighed sample mass placed in an Eppendorf tube was treated with 3 mL of solvent. For the ultrasound assisted extraction procedure two solvents were initially used, i.e. methanol (MeOH) and acetoni-

trile (ACN). Later, MeOH was preferred due to its higher extraction efficacy. After adding the solvent, the samples were subjected to the ultrasound-assisted extraction process in an Elmasonic S70H ultrasonic bath for 45 minutes (3 cycles × 15 minutes), which has been proven to suffice a good extraction yield and not to aggressively degrade the chemical composition of the extract (12). After this step, the Eppendorff units containing the samples extracted into methanol (MeOH) were coated with aluminum foil and allowed to stand in the dark for 24 hours. Immediately after, the Eppendorff units containing the samples were centrifuged for 5 minutes at 5000 rpm at a temperature of 40°C using a Hettich Universal 320R centrifuge. Each generated supernatant was transferred with dedicated Pasteur pipette into new Eppendorf tubes. The obtained extracts were subjected to specific drying procedures using the principle of solid phase extraction (SPE). Homemade cartridges filled with anhydrous sodium sulphate (Na_2SO_4 anh.) and glass wool as stopper were used to dry each obtained extract. The extracts thus generated were passed through Pall Acrodisc PSF, G×F/GHP filters of 0.2 μm porosity. Volumes in the 1-2 mL range were transferred into brown vials and then stored in the refrigerator (4^oC) until the analysis.

Sample analysis. Analysis of the entire set of samples was performed on the GC-MS system at the CERNESIM Center, Integrated Center of Environmental Science Studies in the Northeastern Region, at the "Alexandru Ioan Cuza" University of Iasi, Faculty of Chemistry, Analytical Chemistry Laboratory. Samples were manually injected with a micro syringe into a gas chromatograph, 7890A GC model from Agilent Technologies, equipped with flame ionization detector (FID) and a mass spectrometer 5975C MS model from Agilent Technologies, USA.

For sample analysis the optimized working conditions of the equipment were: 1) helium as mobile phase; 2) capillary column HP-5ms type (30 m length, 0.25 mm internal diameter and 0.25 μm film thickness) as a stationary phase; 3) 270 °C operating temperature of the injector; 4) 0.003 mL injected volume under spitless conditions; 5) temperature gradient with the following steps: 60 ˚C for 1 minute, ramp with 30 ˚C/min. up to 220 ˚C (0 min.), followed by a ramp of 2 ˚C/min to 240 ˚C, constantly 4 minutes, followed by a ramp of 10 ˚C/min to 290 ˚C (constant 10 min.) 6) 230 °C MSD temperature; 7) 150 ˚C quadrupole unit temperature; 8) full scan mode for acquisition of mass spectra; 9) scanned mass range m/z 60-650 atomic mass units; 10) ChemStation, Agilent Technologies soft keys and NIST database for data analysis.

RESULTS

Gas chromatography tandem mass spectrometry (GC-MS) has been used for the analysis of the non-exposed and exposed pesticide matrices. In the phytochemical constituents of both *Golden Delicious* and *Jonathan* matrices, tebuconazole and pesticides present in clearly stated combinational pesticide formulations have been identified upon the sample state, i.e., pesticides non-exposed and exposed matrices.

Attempts have been made to derive chromatographic fingerprints of the investigated samples following the GC-MS analysis of *Golden Delicious* and *Jonathan* leaf and bark extracts from pesticide non-exposed and exposed matrices.

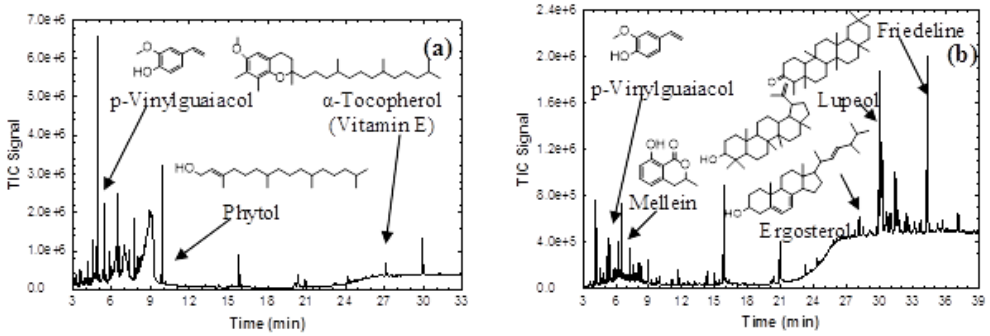

Fig. 1.a,b: Total ion chromatogram (TIC) profiles for pesticides non-exposed *Golden Delicious* matrices: (a) – leaves and (b) – bark methanol extracts.

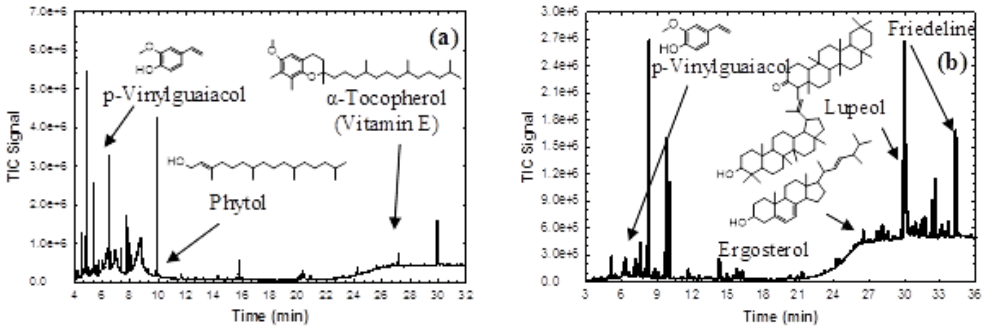

Fig. 2.a,b: Total ion chromatogram (TIC) profiles for pesticides non-exposed *Jonathan* matrices: (a) – leaves and (b) – bark methanol extracts.

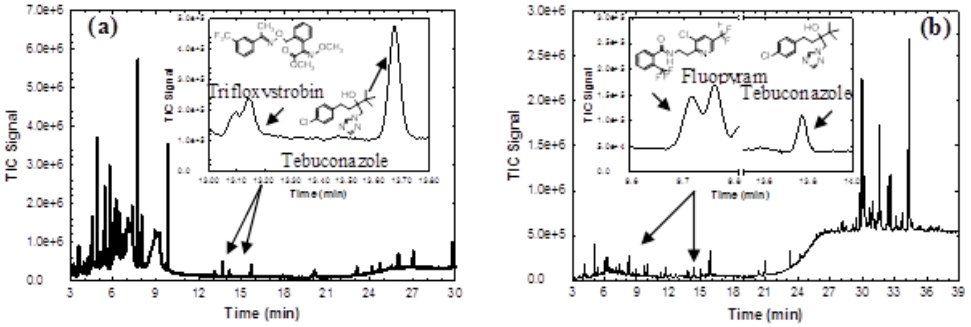

Fig. 3.a,b: Total ion chromatogram frame of methanol extract from pesticides exposed *Golden Delicious* variety: (a) - leaves, (b) - bark. The inset figures highlight in details the chromatographic peaks associated with tebuconazole, trifloxystrobin and fluopyram.

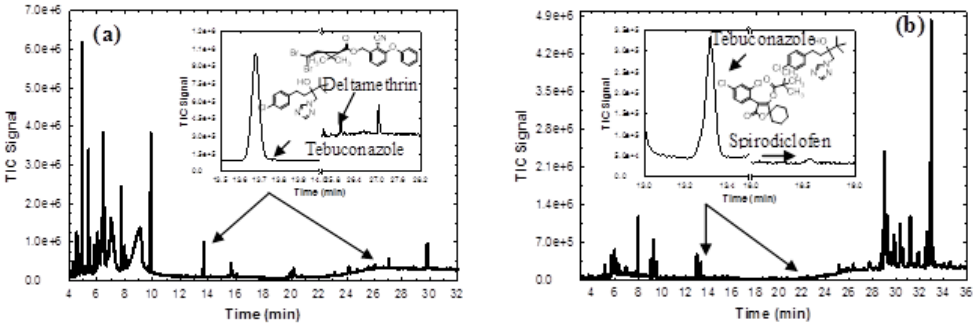

Fig. 4.a,b: Total ion chromatogram frame of methanol extract from pesticides exposed *Jonathan* variety: (a) - leaves, (b) - bark. The inset figures highlight in details the chromatographic peaks associated with tebuconazole, deltamethrin and spirodiclofen.

DISCUSSION

In the present study attempts have been made to derive chromatographic fingerprints of the investigated samples following the GC-MS analysis of *Golden Delicious* and *Jonathan* leaf and bark extracts from pesticide non-exposed and exposed matrices. Figures 1.a,b show some of the main phytochemical constituents (phytol, p-vinyl guaiacol, α-tocopherol/vitamin E) identified in pesticide non-exposed *Golden Delicious* apple leaf (fig.1.a.) and, bark, respectively (fig.1.b.) matrices where compounds such as p-viny guaiacol, lupeol, friedelin, ergosterol, mellein have been identified.

Analysis of the obtained chromatographic profiles highlighted the existence of an important number of bioactive compounds in methanol extracts from both leaves and bark of *Golden Delicious* variety (pesticide non-exposed and exposed matrices) and leaves/bark extracts of *Jonathan* apple variety from pesticide non-exposed and exposed matrices. For sample GC-MS analysis total ion chromatogram (TIC) acquisition mode was preferred to single ion monitoring (SIM) mode and this procedure allowed us to identify a larger array of chemicals. In the investigated pesticide non-exposed matrices several important phytochemical constituents such as methoxyphenol compounds, terpene, tocopherols (α-tocopherol or Vitamin E), phytol, sterols, etc. have been identified.

Chromatographic profiles presented in Figures. 1. a, b and 2. a, b, respectively, clearly highlight features of methanol (MeOH) leave (fig.1.a and fig.2a) and bark extracts (fig. 1.b and fig. 2.b) from pesticide non-exposed *Golden Delicious* and *Jonathan* apple varieties. Regarding the metabolites present in the investigated matrices quite similar information was generated by the investigated samples. However, the abundance of secondary metabolites identified in the analyzed matrices varied from one apple sort to another, i.e. *Golden Delicious* and *Jonathan*. Abundances were determined based on the relationship between the area of each integrated chromatographic pick and the total area of all existent and identified chromatographic peaks (via the NIST database) in a processed signal.

Phytol was identified as one of the most abundant secondary metabolite in both

Golden Delicious and *Jonathan* leaf extracts. Phytol is a secondary metabolite obtained during chlorophylls degradation. It plays an important role in tocopherol synthesis, which is one of the most abundant chloroplast liposoluble antioxidant groups. Analysis of the entire data-base generated by the present study allowed us suggesting the idea about the existence of different metabolic processes in the investigated trees compartments, in full agreement with information from the literature. Phytol is a widespread diterpenoid compound, and recent studies have shown that phytol can act as an excellent immunostimulant with antioxidant, anti-inflammatory and anti-allergic properties (13). Moreover, phytol exhibits also antimicrobial activity against *Mycobacterium tuberculosis* (14) and *Staphylococcus aureus* (15).

α-Tocopherol known also as vitamin E is another important secondary metabolite identified in leaves but not in bark extracts. Vitamin E is the name of all known varieties, referring to a group of compounds exhibiting biological activity, and an example of such a compound is α-tocopherol. It is an active chemical ingredient with one of the highest biological activity in most biological systems. However, the results of recent research studies indicate that there are other vitamin E compounds that can be even more efficient in specific biological mechanisms. Due to its solubility in fat, α-tocopherol has a unique function of protecting cell membranes made of fatty acids against damage by the action of Reactive Oxygen Species (ROS) and is also involved in the photosynthesis process (16).

Another important metabolite identified in leaf and bark extracts of pesticides non-exposed and exposed *Golden Delicious* and *Jonathan* matrices is p-vinyl guaiacol (PVG). It is an important metabolite generated during ferulic acid degradation and is an abundant phytochemical compound found in plant cell wall compartments. The presence of p-vinylguaiacol in the investigated matrices could suggest its potential importance as a phenolic compound existent in various plants' compartments usually known for their high content of antioxidant potential components (17).

In pesticides non-exposed leaves and bark methanol extracts from *Golden Delicious* and *Jonathan* apple varieties, compounds of pharmacological interest, i.e. lupeol, friedelin, α-tocopherol/vitamin E, methoxyphenols, mellein, have also been identified. Introspection of the recorded chromatograms revealed that good separation resolution was achieved for most identified compounds. Although now the information is only of a qualitative nature, the results can then be exploited in establishing strategies for appropriate scientific approaches to elucidate aspects related to the role and implications induced by the presence of pharmacologically relevant metabolites in the investigated matrices.

Lupeol is another secondary metabolite identified in *Golden Delicious* (fig. 1.b) and *Jonathan* (fig. 2.b) bark. It is a triterpene known mainly as fagarsterol and is a compound for which there are suggestions that it would have beneficial effects as a therapeutic and preventive agent for various diseases. Over the past 15 years, much improvement has been made regarding development of this very important molecule for clinical use in the treatment of various disorders. Various studies, focusing on investigating lupeol's action mechanism or its anti-inflammatory potential, have also proven that effective therapeutic doses do not present toxicity to normal cells and

tissues (18). In a research study on lupeol's ester effects it has been shown that this compound posess higher anti-inflammatory properties than indomethacin, with stronger effects in reducing arthritis foot inflammation in rats (19).

In *Golden Delicious* (fig. 1.b) and *Jonathan* (fig. 2.b) bark matrices friedelin was another important secondary metabolite identified. The results of various research studies reported in the literature have indicated that friedelin is very efficient in the treatment of inflammatory diseases, and that it can act as a potential drug with analgesic and antipyretic effect. Synthesis process inhibition and inhibition in the release of inflammatory mediators are considered as important action mechanism for this compound. Due to friedelin's remarkable biological activity presently is strongly believed that it would be imperiously necessary to carry out further research to develop a drug containing this active ingredient (20). In another research study it has been shown that friedelin has also antidiarrheal activity due to its anti-secretory and intestinal anti-motility properties (21).

In the methanol extract of *Golden Delicious* bark (Fig. 1.b) mellein appears as another identified compound. It is an active compound assigned as hepatitis C inhibitor. Mellein compounds are well-known dihydroisocoumarins that are widely distributed in fungi. According to the details in Pontius et al., 2008 (22), mellein was firstly described as a metabolite from *Aspergillus melleus* in 1933. The *Aspergillus ochraceus* extract was found to inhibit the final polyprotein processing step in the hepatitis C virus replication time interval and this fractional bioassay process resulted in the isolation of mellein as an active component of the extract (23).

Vitamin E was identified in *Golden Delicious* (fig. 1.a) and *Jonathan* (fig. 2.a) leaves extracts. Vitamin E antioxidant properties are well known, but in recent years the research interests switch to the potential of this vitamin to be used in pediatrics. It seems that vitamin E supplementation during pregnancy is required especially for α-tocopherol concentration in the colostrum (24) while after a high-risk pregnancy it would be necessary (25).

Analysis of the chromatograms presented in Figure 3.a,b and Figure 4.a,b, respectively, presenting TIC signals of *Golden Delicious* and *Jonathan* methanol (MeOH) leave (fig. 3.a and fig. 4.a) and bark (fig. 3.b and fig. 4.b) extracts clearly revealed the presence of the pesticides of interest in the investigated matrices. Use of an optimized analysis method for the investigation of the matrices of interest by the GC-MS technique has resulted in the identification of tebuconazole at a retention time of 13.75 minutes. In the chromatograms associated with pesticide exposed matrices it was also possible to detect other pesticides present in the in-use clearly stated combinational pesticides formulations. In any case, while for the chromatographic peak associated with tebuconazole the analytical aspects were very clear, it seems that for other compounds the method should be optimized.

Analysis of the chromatographic details specific for *Golden Delicious* bark extracts in pesticide non-exposed and exposed matrices (fig. 1.b and fig. 3.b) allowed us to identify the fact that peak intensity associated with mellein (fungus) gradually diminished after pesticides treatments spread. It is believed that such behavior clearly underlines the potential aggressive effect of treatment components on various promising phytochemical constituent existent in

plant compartments.

CONCLUSIONS

In our study methanol extracts from *Golden Delicious* and *Jonathan* leave, and bark matrices collected from an orchard in Iasi, northeastern Romania were obtained, and a systematic investigation of the existing secondary metabolites was conducted for the first time. Compounds of pharmacological interest, i.e. lupeol, friedelin, α-tocopherol/vitamin E, mellein, have been identified with output creating premises for subsequent scientific approaches in the topic of interest. In the pesticide exposed matrices subjected to GC-MS analyses, there was clear evidence of the presence of tebuconazole and other pesticides in the in-use clearly stated combinational pesticides formulations, containing trifloxistrobin, deltamethrin, fluopiram and spirodiclofen. By monitoring the behavior of mellein metabolite (fungus), mainly existent in the bark of *Golden Delicious* variety, it was possible to highlight the potential aggressive action of the in-use clearly stated combinational pesticides formulations on promising phytochemical constituent existent in various plant compartments.

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