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ORIGINAL PAPERS

STUDY ON THIOPHENES EXTRACTION EFFICACY FROM *TAGETES PATULA L.*

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Abstract. *The species of the genus Tagetes (family Asteraceae) known as ″marigolds″ are usually cultivated as ornamental plants, but are also studied and valued for their pharmacological properties in medicinal practice and for their biological actions in agriculture. The antifungal effect of the extracts, thanks to the content of thiophenes has been demonstrated in numerous studies. The study aims to obtain a qualitative extract from Tagetes patula L. (French marigold) with antifungal properties. The terthiophene in the extract obtained from dried inflorescences of a cultivar of Tagetes patula L. has been assayed by gas-chromatography, based on the calibration curve of the reference substance and has been compared to the content of the vegetal product. The extraction yield of the identified thiophenes, calculated as areas ratio between the extract and the plant, was 78%, while the median recovery ratio of the terthiophene (concentration in plant 0.021%) was 90%. The content corresponds to a significant potential of the antifungal effect of the extract.*

Keywords: Tagetes patula, thiophenes, alpha-terthienyl, extraction, GC-MS, SIM

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Abbreviations

TIC (Total Ion Chromatography), SIM (Selected Ion Monitoring), TBME (*tert*-Butyl methyl ether), GC-MS (Gas Chromatography-Mass Spectrometry), ButG (Butylene Glycol), α-T (alfa-terthienyl, terthiophene), BBT (2-but-3-en-1-ynyl-5-thiophen-2-ylthiophene), PBT (5-(Pent-3-en-1-yn-1-yl)-2,2'-bithiophene), BBTOH (4-(5-thiophen-2-ylthiophen-2-yl)but-3-yn-2-ol).

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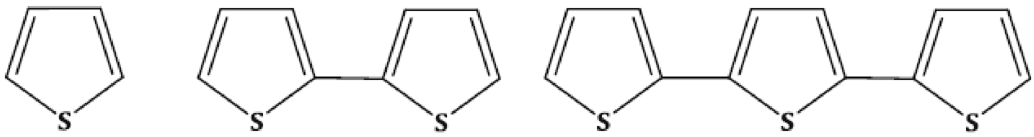
Introduction

The genus *Tagetes* contains more the 50 species (Singh, Gupta and Kannojia 2020). Based on the pharmacological studies, these are used in standardised pharmaceutical formulations (particularly for their content of carotenoids) and have proved their efficacy in the plant protection field as well.

The already published studies demonstrate that the alcoholic extracts from *Tagetes species* (*T.patula*, *T.minuta*, *T.erecta*, *T.tenuifolia*, *T.lucida*), plant and/or root or volatile oil possess antifungal properties (Saha and Walia 2012); (Ibrahim, et al. 2016); (Joshi and Barbalho 2022); (Ali, Jubair and Mohammadali 2020); (Salehi, et al. 2018); (Varahi, Alphiene and Darling 2019) or have insecticidal/larvicidal potential, by phototoxic inhibition of the nematodes (Zannah, Cahyana and Saefumillah 2021); (Marotti, et al. 2010); (Mir, Ahanger and Agarwal 2019); (Sanches, et al. 2014) or even general biocidal effect (Vijayta, Shanker and ur Rahman 2015).

The substances present in the Tagetes plants exert an allelopathic effect to the organisms' rhizosphere, thus the soil nematodes would be annihilated at thiophenes levels under 1 ppm. Research works showed that the biosynthesis of these compounds happens inside the roots, where the thiophene content has been monitored for in vitro generated cultures (Margl, Eisenreich, et al. 2001); (Vijayta, Shanker and ur Rahman 2015); (Breteler and Ketel 1993). Though, the thiophenes have been identified and assayed in variable quantities in all plant organs.

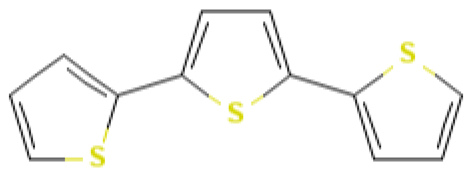
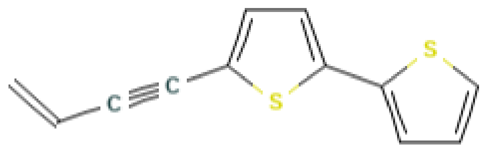
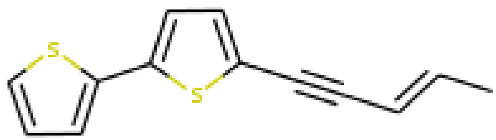
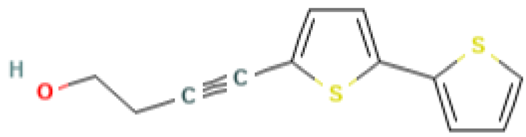
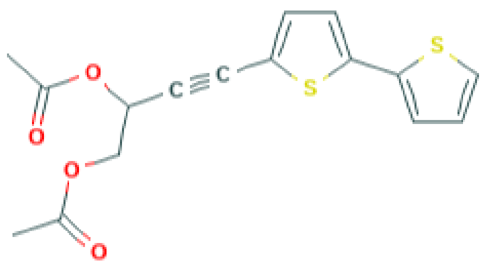
The thiophenes are compounds made up by one or more molecules/radicals of thiophenes/thienyls (aromatic heterocycle with 4 C atoms and 1 S atom).



Thiophene BithiopheneTerthiophene

In the case of lateral carbon atoms of thiophene structure being coupled by alkyl bonds to radicals HO-butynyl, acetoxybutynyl, buten-ynyl, penten-ynyl etc., the resulting compounds are being named acetylenic polythiophenes. This kind of substances have also been identified in *Tagetes* species (Elgahme and Wittstock 2018). The structures of some of these derivates are shown in Table 1.

Table 1. List of some thiophene derivatives cited by literature to be found in *Tagetes patula*

IUPAC name/MW	Structure	Abbreviated name
2,5-dithiophen-2-yl-thiophene (α-Terthienyl) MW=248.4		α-T
2-but-3-en-1-ynyl-5-thiophen-2-ylthiophene MW=216.3		BBT
5-(Pent-3-en-1-yn-1-yl)-2,2'-bithiophene MW=230		PBT
4-(5-thiophen-2-ylthiophen-2-yl)but-3-yn-2-ol MW=234		BBTOH
[2-acetyloxy-4-(5-thiophen-2-ylthiophen-2-yl)but-3-ynyl] acetate MW=334		BBT(OAc)$_2$

Alpha-terthienyl (α-T) is the compound for which the current work desires the quantification. It is a photosensitive and phototoxic compound, with significant insecticidal, larvicidal and fungicidal potential.

The total thiophenes concentration varies with species, organ, harvesting period (Kodithuwakku, et al. 2016), while the compounds distribution varies as well and is represented by combinations of polythiophenes as the ones mentioned in the table above: α-T, BBT, PBT, BBTOH, $BBT(OAc)_2$ and other compounds. For optimal extraction yields, the technological steps should include conditions avoiding light exposure, as thiophenes biological activity is photosensitive (Deineka, et al. 2014).

The following possibilities of thiophenes extraction are cited:

- in the volatile oils' distillation systems Neo Clevenger type, with the disadvantage of selective concentration of the compounds that can be distilled or steam distilled.
- with solvents of solvents mixtures of convenient polarity, for the extraction and concentration of volatile oil phase and other components such as fatty acids and derivatives, tocopherols, sterols, polyphenols, flavones, saponins etc.

The identified methods of analysis in the literature mention the possibilities of photodegradation and volatilization, thus it is recommended to manipulate the extracts in low light intensity conditions (as well as conditioning them in brown or opaque recipients) and to carefully monitor the temperature in extraction and concentration steps. The identification and quantification analyses include chromatographic separation techniques such as GC-MS (Margl, Tei, et al. 2001); (Szarka, et al. 2006); (Saha and Walia 2012), HPLC (Deineka, et al. 2014); (Zannah, Cahyana and Saefumillah 2021); (Vijayta, Shanker and ur Rahman 2015) and HPTLC (Deineka, et al. 2014) or spectrometric techniques such as FTIR (Kodithuwakku, et al. 2016) and UV-VIS (Kodithuwakku, et al. 2016).

Aim of the study

This study aims to evaluate the extraction efficacy of thiophenes from the dried flower heads of *Tagetes patula*, to identify and quantify them, and in the near future to find a suitable and industrially scalable way to obtain a reproductible extract with the following characteristics:

- to possess antifungal properties
- to be stable for at least 1 year
- to be easy to handle and formulate (for further usage and incorporation in a final product)
- to be cost and time effective and environmentally friendly.
-

Material and methods

The solvent used for the micropilot extraction is ethyl alcohol analytical grade, manufacturer Chimreactiv. The solvents used for the extractions for analyses (methanol, n-hexane, TBME) are HPLC grade, manufacturer Carlo-Erba, and the alpha-terthienyl standard (lot F2212) has 99% purity, manufacturer SCBT.

Vegetal material. The vegetal raw material is represented by marigold inflorescences (*Tagetes patula* L.) which were cultivated and harvested in an own production facility in Dâmbovița County, Romania. The flower heads were naturally dried in the dark and conditioned in paper bags; prior to extraction, the material was grounded with the aid of a kitchen robot.

Micropilot extraction procedure for the marigold flower heads has been performed by dynamic percolation with ethyl alcohol 50-70% in three successive steps, at room temperature. The combined plant-to-solvent ratio was 1:27-30 (m/m). The resulted hydroethanolic extracts were concentrated at a rotaevaporator at 110-30 mbar, 50°C, until the solvents are evaporated. The concentrated suspension is included in ButG with 10% ethyl alcohol thus the drug-to-extract ratio is 1:0.8-1.5, centrifuged at 8000 rpm, 20 min, 20°C. The supernatant was separated and conditioned in plastic bottles.

Vegetal product processing for analysis was carried out respecting the protocol described by (Margl, Eisenreich, et al. 2001). The procedure states that 0.2000 g of powdered vegetal product should be extract 3 times with 10 mL each of methanol 70%. The methanolic extracts are filtered and reunited, then extracted by partition 3 times with 10 mL each of a mixture n-hexane: TBME 1:1 (V/V). The non-polar solvents mixture layers are separated, brought together and filtered through anhydrous sodium sulphate directly in the evaporator flask. The sample is concentrated to a volume of 1-2 mL, at 300 mbar, temperature ≤ 45°C and thereafter dried under nitrogen current. The solid sample is quantitatively redispersed in 2 mL of hexane and filtered on PTFE 0.2 µm. The filter is injected in the GC-MS (TIC mode screening). The quantitative analysis of α-T is performed in SIM mode, after a 1:20 dilution of the sample with hexane, by the external standard method, with calibration curve, realized on standard solutions of α-T.

Extract samples processing has been done in order to isolate the thiophenes from the extract conserved in ButG+ethyl alcohol. The extract equivalent to 0.2 g of vegetal material (considering the drug-to-extract ration) was dispersed in 2 mL sodium chloride saturated solution (in order to enhance the partition) and partition with n-hexane/TBME and concentrated the same as above.

Gas chromatography analysis was performed on a Thermo-Scientific equipment, model Trace 1310/TSQ8000-Evo, column TG-5SilMS, 30 m, Φ=0.25 mm, film=0.25 µm, injector splitless, injector temperature 250°C, gas flow 1.2 mL/min, oven temperature program from 40 to 300°C in steps, MS acquisition range 40-600 a.m.u., transfer line temperature 280°C, ionization source temperature 250°C. For SIM analysis, specific fragments for the targeted compounds were chosen as presented in Table 2.

Table 2: SIM specific fragments for thiophenes identification

Name	Retention time (min)	Specific ions		
		Quant	Qual	
BBT	14.15	**216**	171	217
PBT	16.26	**230**	229	231
Tertiofen	18.26	**248**	249	250

Recovery of thiophene by the implemented extraction procedure was calculated using the following formula:

formula:

$$Yield\ of\ extraction\ (\%) = \frac{Det.conc.in\ extract\ (\%)}{Det.conc.in\ veg.material\ (\%)} * \frac{Extract\ mass\ (g)}{Extracted\ veg.material\ mass\ (g)} * 100$$

Results and discussions

For the micropilot extraction, 856 g of dried flower heads of *Tagetes patula* were used, from which 951 g of concentrated extract conditioned in ButG with 10% ethyl alcohol were obtained; drug-to-extract ratio was 1:1.11 (m/m). By taking into consideration this ratio, for the following analyses there were used 0.2 g of vegetal material and 0.22 g of extract per analysis.

TIC screening analysis showed that chromatographic fingerprints of vegetal material extracted samples and extract samples are similar, but with significant variability in the case of volatile compounds peaks, e.g., caryophyllene, piperitenone, caryophyllene oxide etc. These compounds are present in the extract in a proportion less than 10% (0.5% for caryophyllene for example) compared to the vegetal material extracted samples. Thiophenes such as BBT, PBT, BBTOH and α-T have been identified by the GC-MS method (based on the NIST spectral library) in the vegetal material as well as in the extract. Median recovery of the identified compounds is 77.7%. Table 3 presents the recovery calculated levels as areas ratio percent of the identified compounds in extract and vegetal material.

Table 3: List of identified compounds and their proportion recovery in the extract

Peak Name	Retention time (min)	Peak areas ratio extract/plant (%)
Tagetenone	7.971	31.7
Piperitenone	8.789	9.4
Caryophyllene	9.363	0.5
Germacrene D	9.788	1.4
alpha-Farnesene	9.835	28.3
Spathulenol	10.567	34.5
Caryophyllene oxide	10.637	7.3
Myristic acid	12.035	51.5
Isopropyl myristate	12.723	69.9
Neophytadiene	12.901	7.7
Hexahydrofarnesyl acetone	12.984	41.5
Palmitic acid, methyl ester	13.954	44.7
BBT	14.121	79.2
Palmitic acid	14.608	51.4
Palmitic acid, ethyl ester	14.862	95.5
l-(+)-Ascorbic acid 2,6-dihexadecanoate	14.93	49.8
PBT	16.237	62.44
Methyl linoleate	16.301	28.8
Methyl linolenate + oleate	16.378	0.0
Linoleic acid	17.156	1.2
Linoleic acid ethyl ester	17.301	44.3
BBTOH	17.981	76.4
α-T	18.236	92.8
Heneicosane	19.289	66.8
Squalene	26.966	60.9
Hexatriacontane	27.878	67.4
delta-Tocopherol	28.542	23.9
beta-Tocopherol	29.652	19.74
gamma-Tocopherol	29.826	20.2
alpha-Tocopherol	30.715	18.9
Stigmasterol	32.096	28.2
gamma-Sitosterol	32.834	49.4
beta-Amyrin	33.287	33.8
alpha-Amyrin	33.914	42.9
gamma-Sitostenone	34.655	42.1

The chromatogram associated to the list in Table 3 is presented in Figure 1:

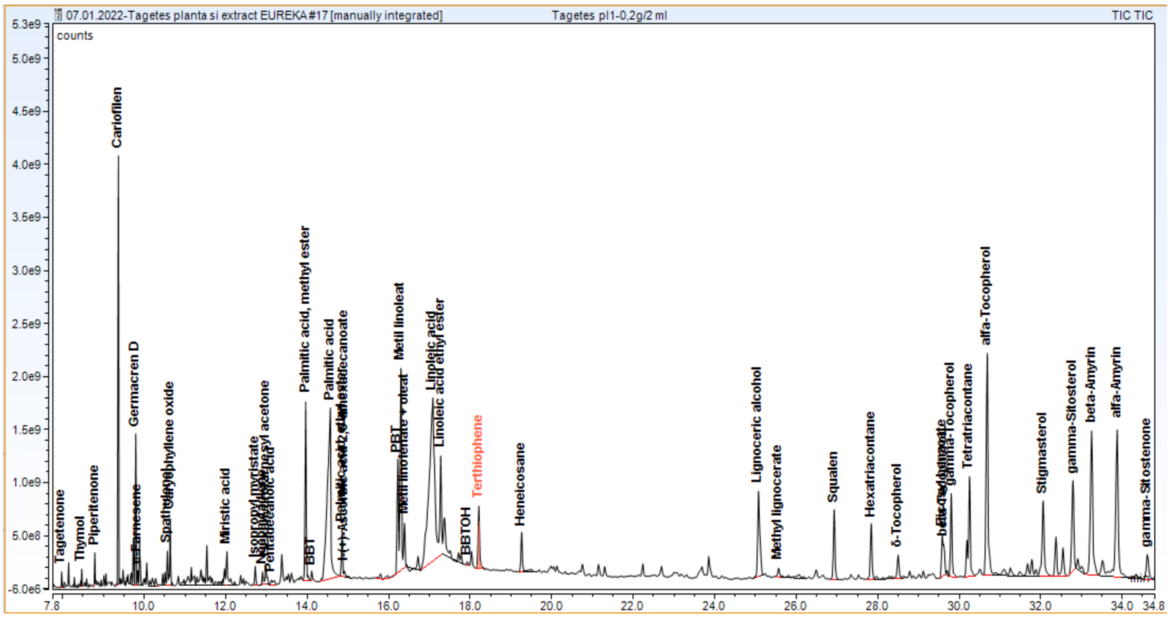


Fig.1 Chromatogram of the compounds present in the Tagetes extract

Two examples of chromatogram overlap for one volatile compound (piperitenone) and one thiophene compound (α-T) from the two types of samples (blue – extract; black – plant) are presented in Figure 2:

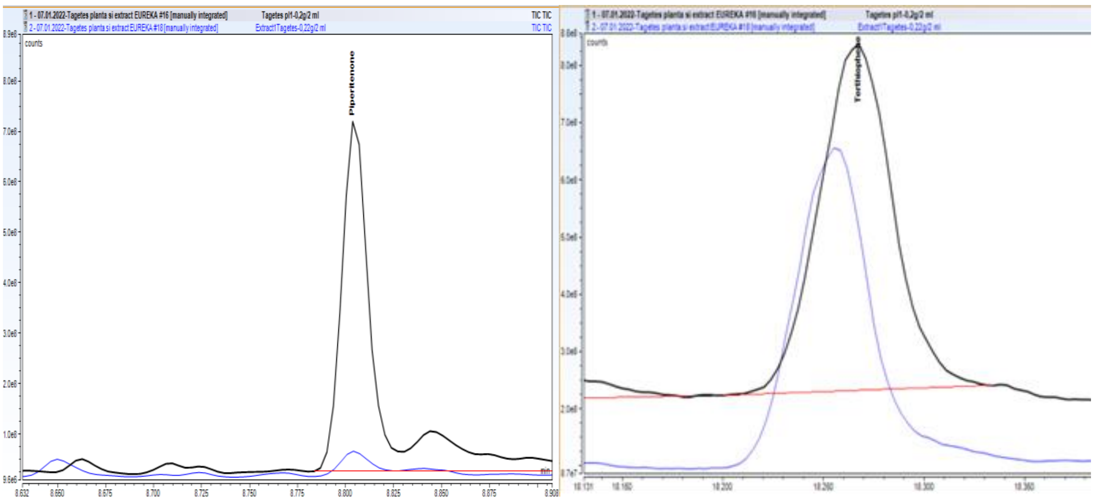


Fig. 2 Overlapped chromatograms (plant/extract) for piperitenone and α-T

For the SIM analysis, details about the acquired ion-chromatograms and the compounds structures are presented in Table 4. The external standard calibration curve for α-T was performed for an interval of 0.4-1.2 µg/mL, with a correlation coefficient R2=0.997 and is shown in Figure 3.

For the duplicate quantitative analysis of the two types of samples, using this method, there were acquired the following median values: 0.021% for the vegetal product and 0.017% for the extract.

The other compounds (volatile oil compounds such as terpenes and the other thiophenes and fatty acids and their esters) have been identified by using the NIST spectral library, but have not been quantitatively assayed.

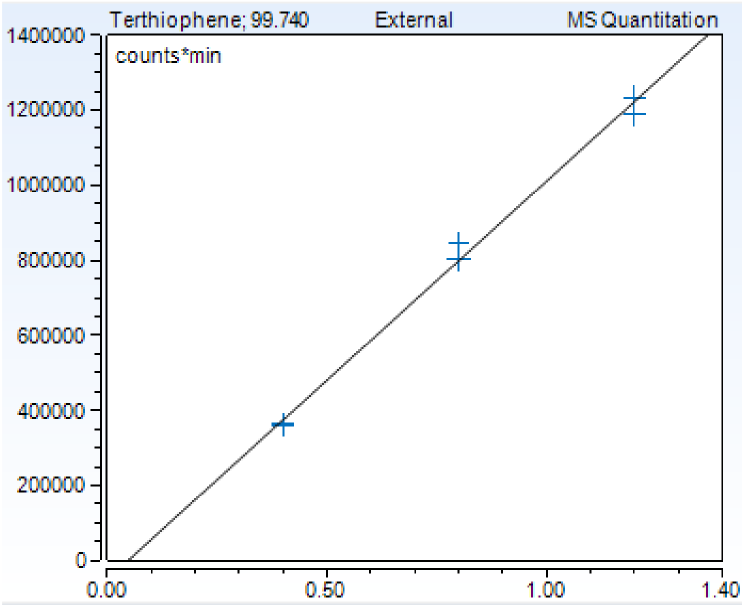
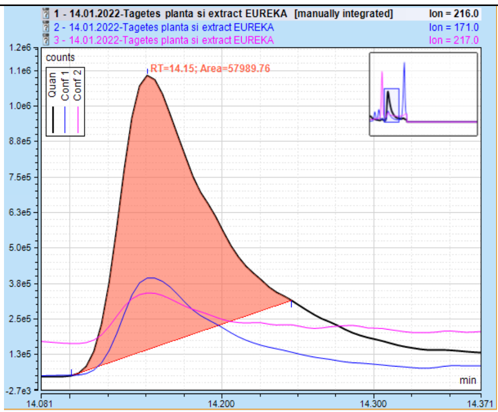
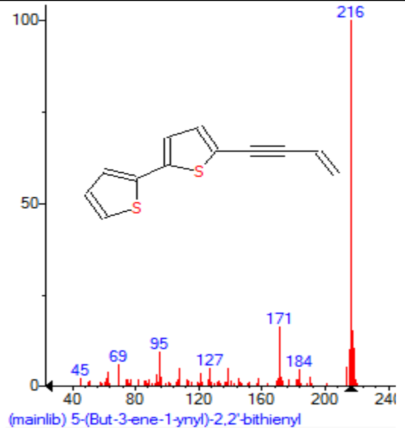


Fig.3: Calibration curve for α-T

Table 4: Ion-chromatograms and spectral structure of some identified compounds in Tagetes patula dried flower heads and extract

<table>
<tr><th>Name</th><th>Ion-chromatogram</th><th>Spectrum and chemical structure</th></tr>
<tr><td>BBT</td><td>
</td><td>
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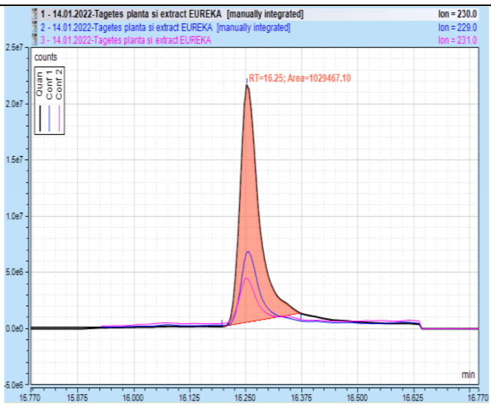
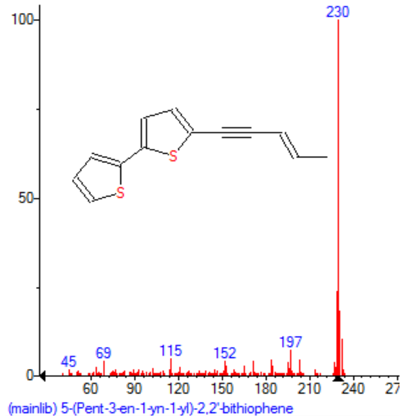
<tr><td>PBT</td><td>
</td><td>
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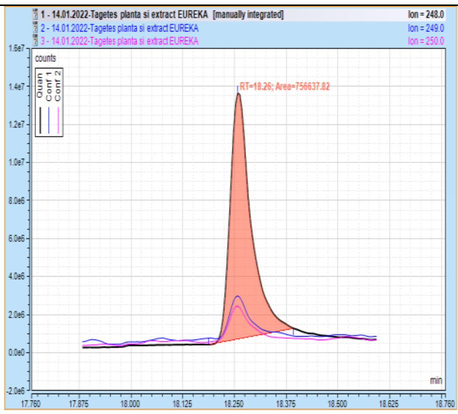
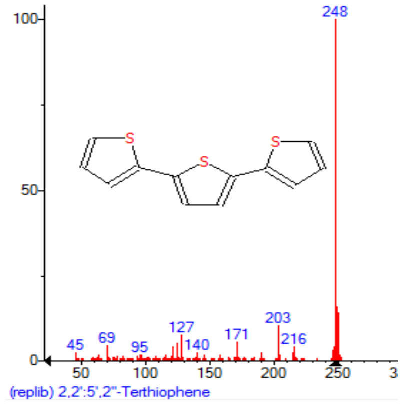
<tr><td>Terthiophene</td><td>
</td><td>
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The extraction yield of α-T is calculated by referring to the concentration determined on the extract to the one determined practically on the vegetal material, considered the theoretical concentration of α-T in the dried vegetal material. The formula above was used and the yield is 90%.

For BBT si PBT, by dividing the areas of the quantification ion (SIM method) from the extract samples and vegetal material samples, the acquired yields (recoveries) are 82% and 72% respectively.

Conclusions

The species Tagetes patula, particularly its flower heads (Tagetes patulae flores, French marigold flowers), by the diversity of the compounds identified in the extract (α-T, PBT, BBT, BBTOH, as well as sterol derivatives, tocopherols and fatty acids and their derivatives) is a phytocomplex with a valuable antifungal potential.

The gas chromatographic method (SIM method particularly) through its sensibility and specificity allows the analysis of very low levels of compounds, from 0.4 µg/mL (0.4µg/5 mg plant material) equivalent to 0.008 % in the vegetal product, which corresponds to the trace levels that thiophenes are usually found in plants.

The transfer ratio of the bioactive complex of thiophenes from the vegetal material to the extract (practically the extraction yield) is 80% on average, for α-T the value is 90%, obtained through the calibration curve method.

Results can be considered as a step forward in the multifactor process of an innovative bio-pesticides development, for the orchards protection. We also aim a proper characterization of the final products' active principles and further structure – activity correlations.

Acknowledgements

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