



E-ISSN: 2278-4136

P-ISSN: 2349-8234

www.phytojournal.com

JPP 2021; 10(1): 175-180

Received: 26-10-2020

Accepted: 08-12-2020

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Rapid and feasible TLC screening of tea tree oil commercial samples

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DOI: <https://doi.org/10.22271/phyto.2021.v10.i1c.13304>

Abstract

Tea tree oil is an essential oil used for medical and health purposes consisting of a complex mixture of organic compounds present in high or trace amounts. Several factors (i.e. chemotype, environmental conditions, preparation procedures) can affect the composition and consequently the biological properties, therefore, quality assurance is a key requisite to guarantee reproducibility among batches. Compliance with ISO standards by gas-chromatography mass-spectrometry techniques are universally accepted in check of tea tree oil formulations, while adulterations might be overlooked. In this study we set a TLC protocol for the easy and cheap evaluation of tea tree oil batches purchased from the marketplace and, coupled to the European Pharmacopoeia assay of fatty oils, for the detection of adulterations. This protocol aims to be a procedure for the quality evaluation of tea tree oil in small laboratories not equipped with expensive analytical instruments and by health professionals dealing with herbal medicines.

Keywords: Tea tree oil, TLC analysis, adulteration, fatty oils, GC-MS analysis

1. Introduction

In the last years, the use of essential oils (EOs) as well as other herbal products has been increasing in popularity as integrative health practice^[1]. Even if EOs are complex natural mixtures of hundreds of components in low or trace amounts, they are usually characterized by two or three components at fairly high concentrations (20-70%)^[2]. Both prevailing and minor components are affected by several factors such as chemotype, environmental conditions, and preparation procedures thus resulting in high variability of the final product^[3]. Quality assurance is an essential prerequisite to guarantee reproducibility among batches as well as safe and effective use^[4]. At present, the International Standard Organization (ISO 4730:2017)^[5] is the main certifying body publishing universally accepted standards for essential oils^[6]. The observance of ISO standards is mandatory to ascertain the quality of EOs, to ensure homogeneity in composition, to precisely evaluate plant material, chemotypes, provenance, and to individuate reconstructed essential oils or storage-associated alterations. Nevertheless, even if EOs match the ISO standards, inherent variability can occur in the undefined and/or trace elements of the mixture. Adulteration is a major issue in the essential oil industry as the optimization of profits pushes the use of cheaper materials in addition to natural essential oils, such as synthetic material, essential oils or vegetable oils^[7].

Among commercially available EOs, tea tree oil (TTO) is widely used both for medical purposes and personal care for its well-known antiseptic properties^[8]. TTO derives from the Australian native plant *Melaleuca alternifolia* Cheel (Myrtaceae). Six different chemotypes have been recognized in *Melaleuca alternifolia*; chemotypes produce oil with distinct chemical composition and are classified by their content in terpinen-4-ol, cineol and terpinolene^[9-11]. Terpinen-4-ol chemotype has been consistently identified as the most effective for the antimicrobial effect, properly due to terpinen-4-ol^[8]. Recently, we evaluated the content of the main constituents and the antimicrobial activity of ten different batches of TTOs (T1-T10) purchased from Italian market. We reported that the tested batches matched international standards, but differences in the content of terpinen-4-ol have been pointed out, and not related to their antimicrobial activity^[12].

Although the major components of EOs are known and play an important role in the EO biological activity, minor components and/or adulterants could be involved through antagonistic or synergistic effects. As regard the TTO, quality control of commercial products aims to verify whether they match the ISO standards, however the possible co-presence of adulterants is not always taken into account^[13-16]. In this respect, the quality control of essential oils remains an open issue.

In this study, we set a TLC method coupled with densitometric analysis for evaluation of TTO batches purchased from the marketplace. We have shown that the TLC profile and a very simple European Pharmacopoeia [17] assay to detect fatty oils proved to be effective in revealing potential adulterations. This study provides a practical, simple, versatile, and economically viable approach in preliminary quality evaluation of tea tree oil based on a "simple to read" chromatographic profile.

2. Materials and methods

2.1 Materials

Ten tea tree oil samples (T1-T10) were purchased from Italian market. T1, T2, T3, T5, T6, T10 come from Australia; T7 originated from Africa; the origin was not specified for T4, T8 and T9. As labeled in the original package, all the batches are stated as oil from *Melaleuca alternifolia*. "100% tea tree oil" was indicated for T1, T2, T3, T5, whereas the other batches simply reported "essential oil". Specimens of all samples are deposited at the Department of Pharmaceutical and Pharmacological Sciences, University of Padua (Italy). Triisopropylbenzene and terpinen-4-ol were purchased from Sigma-Aldrich.

2.2 TLC analyses

Analyses were performed on silica gel 60 F254 precoated aluminum and glass backed plates (Merck). Sample solutions (10 μ L) were applied as spots and the plates were developed with 10 mL of mobile phase (toluene: ethyl acetate 95:5, toluene: ethyl acetate 85:15, cyclohexane: ethyl acetate v/v). Terpenes were detected by spraying with vanillin-sulfuric acid reagent, followed by heating at 105°C for 10 min. The plate images were acquired with an Epson V200 Photo flatbed scanner and elaborated using the Image J image-processing program (available online and developed by the National Institutes of Health, USA).

For linearity evaluation, aliquots of terpinene-4-ol standard solution and of T1 sample were diluted to obtain the working solutions that were then applied to TLC plates to prepare calibration curves. The peak areas and the corresponding amounts were subjected to linear least square analyses. The method was validated for repeatability and intermediate precision (intra- and inter-day precision). Repeatability was proved by multiple measurements (n=4) of terpinene-4-ol standard solution and T1 sample to two concentration levels and was expressed as percent coefficient of variation (% CV).

Intermediate precision was studied by analyzing aliquots of terpinene-4-ol standard solution and T1 sample on the same day (intra-day precision) and on different days (inter-day precision). The results were expressed as percent relative standard deviation (% RSD) between different days. Sample T4 was appropriately diluted with toluene and the solution loaded as a band onto a 20x20 cm analytical plate silicagel 60 F254 glass backed. The plate was developed and dried at room temperature. The silica gel corresponding to the band with Rf 0.75 was carefully scraped with a spatula and eluted with toluene.

2.3 GC-MS analyses

GC-MS analyses were carried out using a Trace GC Ultra (Thermo Fischer Scientific) equipped with a 30 m x 0.25 mm i.d. x 0.25 μ m film thickness Supelco SLB-5 ms fused silica with helium as carrier gas. The GC oven temperature was initially set at 50°C for 4 min; then ramped at 3°C min⁻¹ to 70°C, at 10°C min⁻¹ to 130°C, finally at 3°C min⁻¹ to 150°C and kept at this temperature for 1 min, and finally ramped at 25°C min⁻¹ to 250°C and kept at this temperature for 1 min. Analyses were performed in splitless mode.

Ten microliters of TTO samples were diluted in 10 mL of *n*-hexane. Triisopropylbenzene was added as internal standard to each sample solution at a constant concentration. Duplicate injections were made for each sample.

2.4 Determination of fatty oils in essential oils according to European Pharmacopoeia

One drop of TTO samples has been allowed to fall onto filter paper. The filter paper must be observed after 24 h; the detection of translucent spots reveals the presence of fatty oils [17].

3. Results and Discussion

In a previous study we evaluated the antimicrobial activity of ten different batches of TTOs (T1-T10) purchased from Italian market. Although all the studied TTO batches followed the ISO 4730-2017 guidelines (Tab. 1), differences are pointed out in their activity and not due to the content of terpinen-4-ol. T1 and T7 showed the highest content of terpinen-4-ol; nine batches contained approximately 15% less and T4 40% less than T1 and T7. However T1 and T7 showed minor effects in controlling growth of bacteria and fungi whereas T4 exhibited notable antimicrobial activity [12].

Table 1: Comparison with ISO standards of the major terpenes (%) in the TTO samples

	ISO	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
α -pinene	1,0-4,0	2,4	3,2	5,5	2,9	2,8	3,0	2,2	3,8	6,4	6,2
α -terpinene	6,0-12,0	8,8	10,4	8,5	9,7	12,1	10,7	10,2	6,5	8,9	11,2
<i>p</i> -cimene	0,5-8,0	7,3	7,6	9,6	7,5	5,5	7,0	4,8	9,5	4,2	5,5
limonene + 1,8-cineole	0,5-11,5	4,2	4,4	4,0	3,6	4,7	6,3	3,2	3,7	7,9	3,5
γ -terpinene	14,0-28,0	20,1	23,00	17,0	19,2	25,0	22,8	20,6	20,0	21,4	21,2
terpinolene	1,5-5,0	3,7	3,5	4,3	3,0	4,1	3,9	3,3	2,1	2,8	3,1
terpinene-4-ol	35,0-48,0	47,9	42,4	43,1	41,9	41,2	40,5	47,9	46,1	40,6	40,5
α -terpineol	2,5-5,0	3,8	4,5	4,2	6,5	2,9	3,1	3,5	8,3	7,7	8,2
n.i.	-	1,7	0,8	3,8	5,6	1,9	2,0	4,3	0,0	0,0	0,0

Based on these results, we determined the monoterpene content by GC-MS, the most widely use technique for the analysis of EOs, across the batches looking for comparative indications among the different samples. Therefore, the respective normalized areas in each sample were compared taking the highest area value at 100% and calculating the percentage values for the other samples. As reported in Fig.1,

the difference of the normalized area was less than 20% in nine samples but about 40% in T4 in respect to T10 (100%). Data suggested that sample T4 was diluted and added to other compounds responsible for its activity.

In order to identify likely adulteration, we investigated and developed a rapid and feasible TLC approach in screening of tea tree oil.

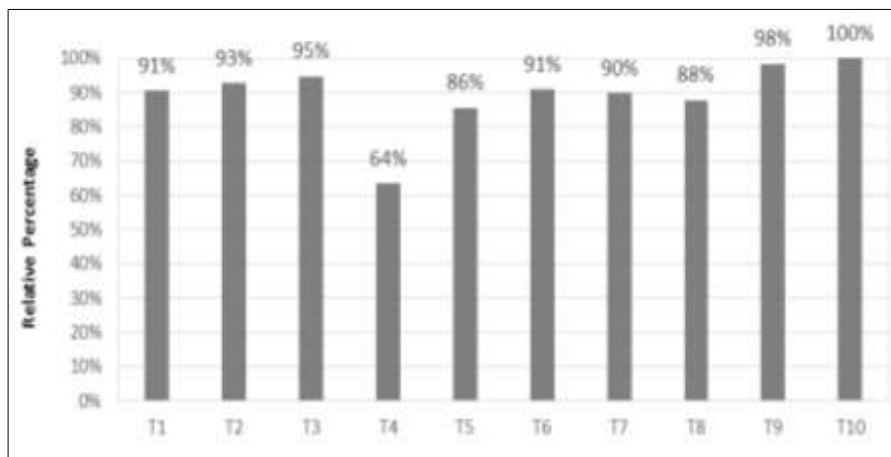


Fig 1: Monoterpene content

To achieve adequate separation of terpinene-4-ol from other TTO components that can be visualized by derivatization, three solvent systems were initially tested as mobile phase. Toluene: ethyl acetate 85:15 v/v reported the best resolution and was selected for the analyses. Under these chromatographic conditions Rf value of terpinene-4-ol was 0.46 with no interferences by other compounds in the sample. Subsequent analyses were performed to determine optimal concentration and volume of samples to be loaded onto the plates. Spots of 10 μ L of a commercial sample (T1) and terpinene-4-ol were loaded at different concentrations: 0.25, 0.5, 1.0, 1.5 and 2.0% (v/v). The best resolution was achieved with 1.5% for T1 and 0.25% for terpinene-4-ol.

Calibration curves were prepared with proper dilutions of terpinene-4-ol standard solution and T1. The plate images were acquired with a flatbed scanner and elaborated using the

Image J image-processing program. The correlation coefficients for linear relationship between concentrations and peak areas were, respectively, 0.9855 and 0.9949, thus indicating good linear relationship between concentrations and peak areas. Repeatability and intermediate precision studies were performed using terpinene-4-ol standard solutions and T1. The values for repeatability were 2.1 and 2.7 (% CV), intra-day precisions were 1.0 and 1.5 (% RSD), and inter-day precisions were 2.0 and 2.8 (% RSD), respectively.

Keeping into consideration the obtained results, TLC was set up for T1-T10 samples. At a glance, it was evident that all the TTO samples belonged to the terpinene-4-ol chemotype (Rf = 0.46). In addition, one spot at Rf = 0.59 and one spot at Rf = 0.31 were evident (Fig. 2) and in agreement with published data they are consistent to 1,8-cineole and α -terpineol [18].

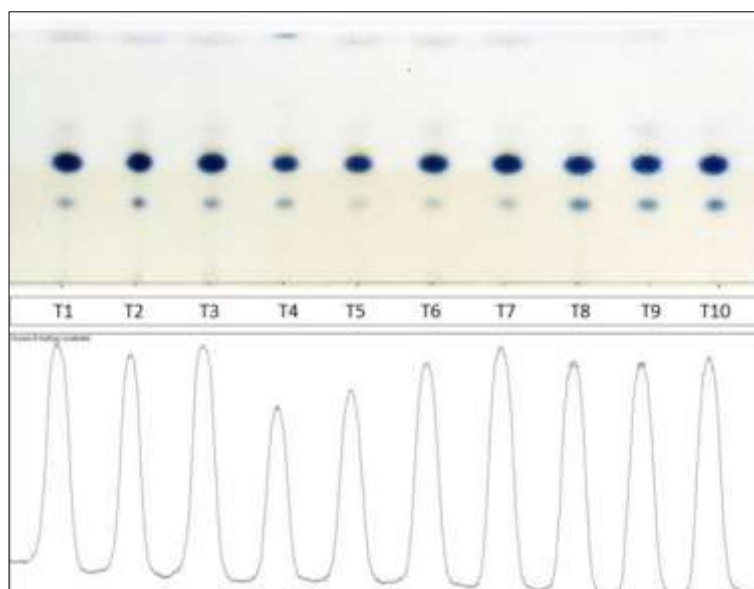


Fig 2: Color image under visible light of TLC plate with T1-T10 samples, after derivatization with vanillin-sulphuric acid, and densitogram obtained with ImageJ software from plate image with integration of terpinene-4-ol peak

The same samples were analyzed by GC-MS; the terpinene-4-ol normalized areas in each sample obtained by TLC and by GC-MS analyses are reported in Figure 3.

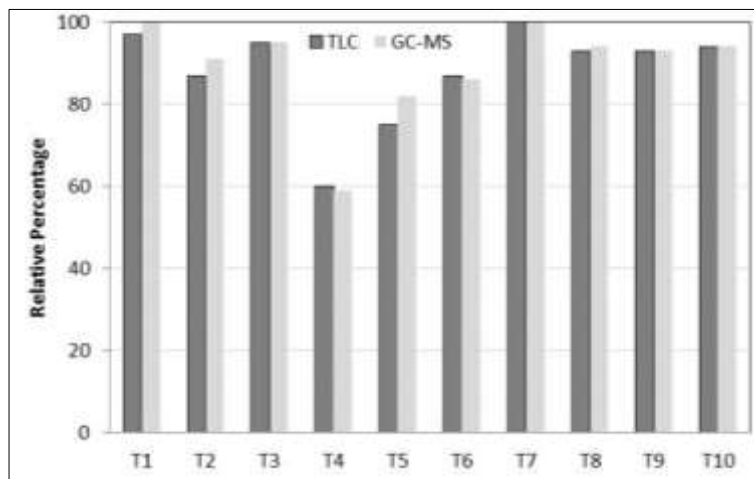


Fig 3: Relative intensity obtained by TLC and by GC-MS of terpinen-4-ol in tea tree oils (T1-T10) taking the highest area value to be 100%

Data were determined taking the highest area value at 100% and calculating the percentage for the other samples. The results obtained by TLC were comparable to data obtained by GC-MS analysis, and the difference of the normalized areas was smaller than 5%.

In order to reveal any compound occurring in small amount, analysis was performed using glass-supported plates allowing the loading of higher volumes of sample. T1-T10 samples were separated on the plate using 4-fold higher concentrations than those used in the quantitative analysis. Under these conditions, chromatogram of T4 reported a marked pink spot (PS) with Rf 0.75, and an intense spot at Rf 1, suggesting the presence of additional lipophilic compounds not detected in the other TTO batches (Fig. 4).

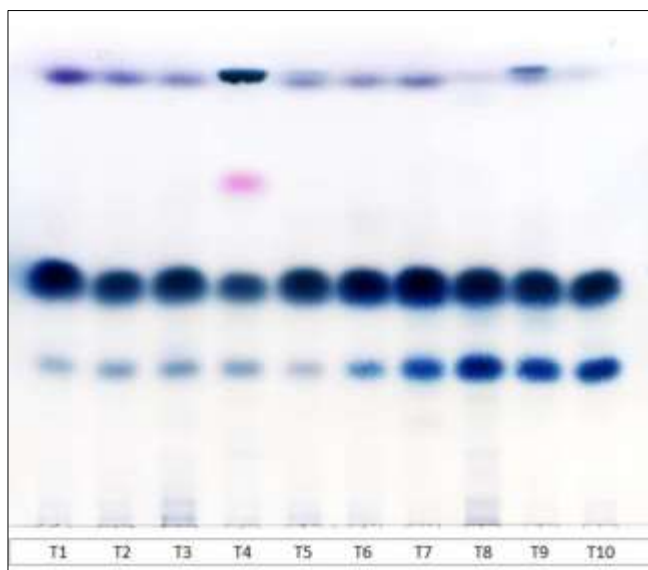


Fig 4: Color image under visible light of TLC plate silica gel glass backed, with T1-T10 samples, after derivatization with vanillin-sulphuric acid. The plate was developed with toluene: ethyl acetate (85:15).

According with previously reported data [18], Rf values and color of the spot indicate the presence of thymol or carvacrol in T4. In order to confirm the identity of analyte, the adsorbent not derivatized layer corresponding to the PS was scraped and eluted from the adsorbent phase using toluene. The solution was then subjected to TLC with thymol and carvacrol as reference standards. Firstly, the Rf of the PS was

very closed to that of thymol and slightly higher than carvacrol, but the difference was too small to be discriminating. Further evidence in support to our hypothesis was obtained from the difference in thymol and carvacrol Rf values obtained by decreasing the polarity of the eluent system. Moreover, after just a few minutes the pink color of carvacrol spot became pale and decreased to almost nil after two hours, in contrast to the PS and thymol spots that reported only partial color decrease (Fig. 5).

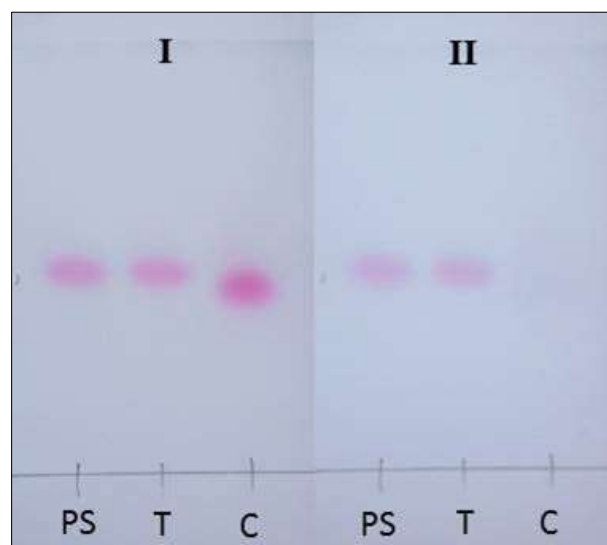


Fig 5: Color image under visible light of TLC plates with isolated compound (PS), thymol (T) and carvacrol (C), immediately after the derivatization (I), and 90 min later the derivatization (II) with vanillin-sulphuric acid. The plate was developed with toluene: ethyl acetate (93:7).

It's known that terpenes produce characteristic colors upon application of the vanillin/H₂SO₄ spray that usually change after 30 minutes at room temperature and frequently further change after a four-hour period. The color of spots and their changes were characteristic and reproducible [19,20].

These findings are strengthened by the higher antimicrobial activity of T4 as compared with other tested TTO batches and by the well-known antimicrobial activity of thymol [21]. Finally, the GC-MS chromatogram of T4 reported a peak at RT=18.04, absent in the chromatograms of the other TTO batches, with MW=150 value in agreement with thymol mass (Fig. 6).

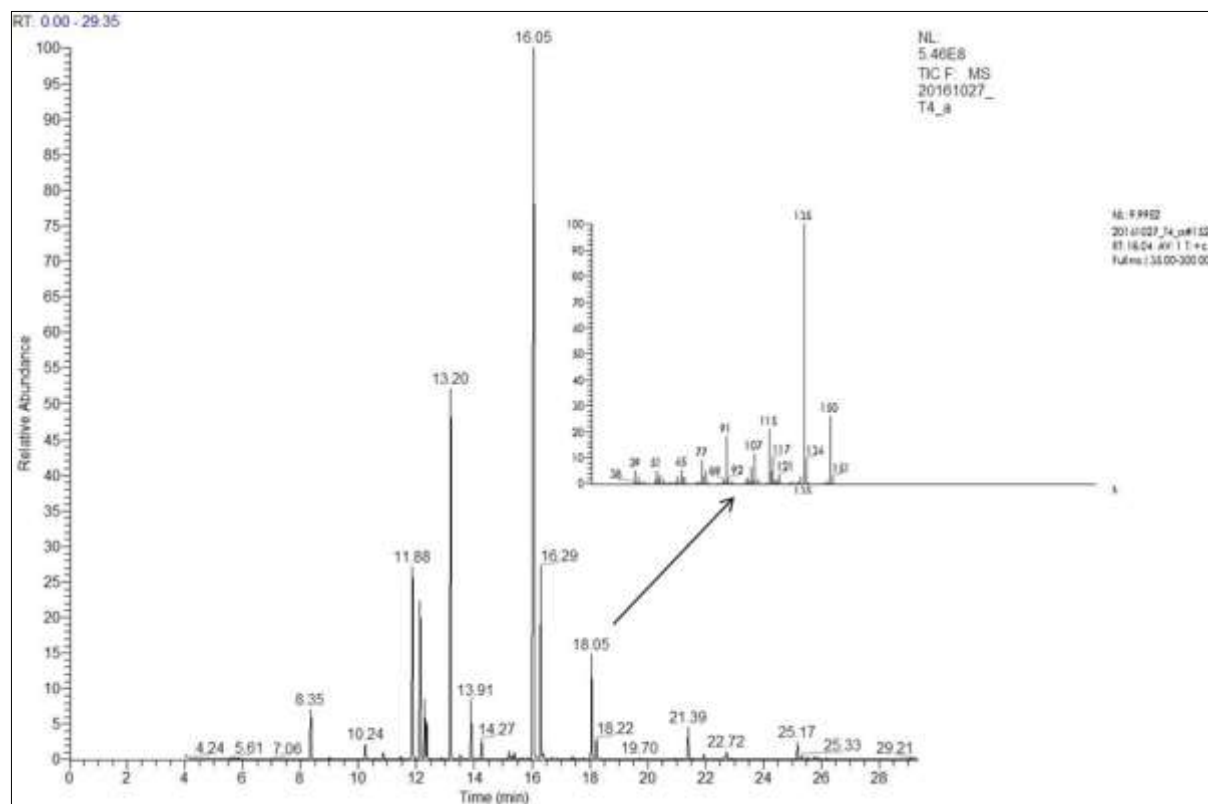


Fig 6: GC-MS of T4 sample and MS spectrum of peak at 18.05 min

As described, beside the PS the chromatogram of T4 reported a spot at Rf 1 ascribing to lipophilic compounds. Indeed, addition of fatty oils is one of the most common methods of essential oil adulteration. Assessment of adulteration of T4 was performed following the indications reported in the European Pharmacopoeia by depositing a drop of samples onto a filter paper. Twenty-four hours later, a translucent spot indicating the presence of fatty oils was evident.

Based on these results, we felt our initial hypothesis was confirmed and T4 revealed the presence of thymol as spiking with pure thymol or *Thymus* spp. in the batch. The presence of thymol in T4 could explain the higher antimicrobial activity, despite its low terpinen-4-ol content. Indeed, a huge body of literature certifies the biostatic and biocidal activity of thymol against Gram-positive and Gram-negative bacteria and fungi reporting MIC values comparable to the data we observed in T4 [12, 21, 22]. Moreover, thymol has been reported to increase generation of reactive oxygen species and respiratory burst in immune competent cells, a mechanism of action we described associated to antimicrobial effect in *Candida* spp [12, 23].

4. Conclusions

Among the essential oils, TTO is one of the most used and common product in the market in the form of both pure product or ingredient in various formulations. Since several uncontrollable factors impact on the composition of prevalent and secondary components of TTO, quality control and adulteration assessment of commercially available formulations is mandatory but lack of reliable and at the same time simple and inexpensive analytical methods.

In this work we have proposed a conventional TLC method for the control of TTO samples. Indeed, by coupling the TLC analysis with image-processing program (i.e. *Image J*) it was possible to compare the terpinen-4-ol content in ten different TTO batches with high reproducibility, rapidity of execution,

and cost-effectiveness. Furthermore, it was possible to reveal in one of the samples the presence of thymol and of fixed oil. Currently, the most widely used technique for the analysis of EOs is GC-MS. Alike GC-MS, TLC plays an important role in the qualitative and quantitative analysis of compounds present in multicomponent mixtures and even if it is not considered the technique of choice, TLC has in many cases some advantages over other chromatographic techniques [24-26]. Indeed, our results show that TLC is an immediate, simplest and feasible analytical tool to check the presence and to identify known compounds and/or adulterants in TTOs. This analytical procedure could usefully be used by small laboratories not equipped with expensive analytical instruments and by operators and health professionals dealing with herbal medicines.

5. Acknowledgements

This work was financially supported by grants from the University of Padova (BIRD/ex-60%).

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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