1. Purpose

Tea tree oil (TTO) is the essential oil of tea tree (Melaleuca alternifolia or M. linariifolia, Myrtaceae). Adulteration of TTO has become more apparent in recent years. Adulteration occurs with single essential oil components (e.g., sabinene from pine oil), waste products derived from other essential oils such as pine (Pinus spp., Pinaceae), eucalyptus (Eucalyptus globulus and other Eucalyptus spp., Myrtaceae), and camphor (Cinnamomum camphora, Lauraceae) oils, or with essential oils from other Melaleuca species and the closely related genus Leptospermum. This Laboratory Guidance Document presents a review of the various analytical technologies used to differentiate between authentic tea tree oil and essential oils containing adulterating materials. This document can be used in conjunction with the Tea Tree Oil Botanical Adulterants Bulletin published by the ABC-AHP-NCNPR Botanical Adulterants Prevention Program in 2017.1

2. Scope

The various analytical methods were reviewed with the specific purpose of identifying strengths and limitations of the existing methods for differentiating tea tree oil from its potentially adulterating materials. Less emphasis will be given to authenticate whole, cut, or powdered tea tree leaves and distinguish them from potential confounding materials by macroscopic, microscopic and genetic analysis. Analysts can use this review to help guide the appropriate choice of techniques for qualitative purposes. The suggestion of a specific method for testing TTO materials in their particular matrix in this Laboratory Guidance Document does not reduce or remove the responsibility of laboratory personnel to demonstrate adequate method performance in their own laboratories using accepted protocols outlined in the United States Food and Drug Administration’s Good Manufacturing Practices (GMPs) rule (21 CFR Part 111) and those published by AOAC International, International Organization for Standardization (ISO), World Health Organization (WHO), and International Conference on Harmonisation (ICH).

3. Common and Scientific Names

3.1 Common name: Tea tree

Note: According to the American Herbal Products Association’s Herbs of Commerce, 2nd ed.,2 the standardized common name of M. alternifolia is tea tree. Melaleuca linariifolia, although rarely used for TTO production, is another accepted source material for TTO according to the ISO,3 but is not listed as such source in the second edition of Herbs of Commerce.
3.2 Other common names for Melaleuca alternifolia

**English:** paperbark tree, narrow-leaved paperbark

**Chinese:** Húsìng yè bái qiān céng (互生叶白千层)

**French:** Mélaleuca (arbre à thé)

**German:** Teebaum

**Italian:** Melaleuca

**Spanish:** Árbol de té

**Latin binomial:** Melaleuca alternifolia (Maiden & Betche) Cheel

**Synonyms:**
- Melaleuca linariifolia
- Melaleuca dissitiflora
- Melaleuca viridiflora
- M. minutifolia
- M. myrtifolia
- M. alba
- M. leucadendra; M. quinquenervia; M. cajuputi, subsp. cajuputi, subsp. platyphylla; M. armillaris, and M. ericifolia

**Botanical family:** Myrtaceae

**4. Botanical Description and Geographical Range**

Melaleuca alternifolia is an evergreen tree native to Australia, where it is endemic to the East coastal littoral of continental Australia from Maryborough in the north to Port Macquarie in the south and west to the Great Dividing Range. The native habitat of M. alternifolia is low-lying, swampy, sub-tropical, coastal ground. Botanical descriptions have been published by a number of sources, Melaleuca alternifolia has been introduced and cultivated in China, Indonesia, Kenya, Madagascar, Malaysia, South Africa, Tanzania, Thailand, United States, and Zimbabwe.

Melaleuca linearifolia is a tree or shrub growing up to 10 m (32.8 ft.). It has a more limited distribution range, being endemic to the Australian states of Queensland and New South Wales, where it is mostly found in coastal areas. It grows in swampy shrub land or open forest, low shrubby or dry sclerophyll forests, eucalyptus woodlands, and on sandy and sandstone soils.

**5. Adulterants and Confounding Materials**

Hagers Handbuch der Pharmazeutischen Praxis lists additional Melaleuca species as potential sources of essential oil, i.e., M. decora, M. dissitiflora, M. quinquenervia, and M. viridiflora. Melaleuca viridiflora, which is the source for niaouli oil, is also listed in Herbs of Commerce. Melaleuca ericifolia essential oil is a relatively limited boutique product and is sold at a premium price compared to TTO; so, it is not a likely adulterant. Melaleuca dissitiflora is indicated as another source for TTO in the European Pharmacopoeia, but is no longer permissible according to the latest ISO guidelines. However, there is currently no evidence that adulteration with these Melaleuca species is an issue in the marketplace.

Sections 6-10 of this document discuss macroscopic, microscopic, organoleptic, genetic, and phytochemical authentication methods for M. alternifolia. A comparison among the various chemical methods is presented in Table 2 on page 6 of this document.

**6. Identification and Distinction using Macroanatomical Characteristics**

Botanical descriptions of tea tree leaves have been published in a number of papers and books. Criteria to distinguish M. alternifolia from other Melaleuca species (M. leucadendra; M. quinquenervia; M. cajuputi, subsp. cajuputi, subsp. platyphylla; M. armillaris, and M. ericifolia) have been published by Barbosa et al. For obvious reasons, macroscopic analysis is not applicable to TTO.

**7. Identification and Distinction using Microanatomical Characteristics**

Two references with details on microanatomical features of M. alternifolia leaves have been retrieved. Shah et al. also published microscopic characteristics of M. leucadendra. Images of cross-sections of leaves and petioles to distinguish tea tree leaves from those of other Melaleuca spp. are provided in the publication by Barbosa et al. Based on the available information, M. alternifolia is readily distinguished from E. globulus using botanical microscopy, e.g., according to the drawings provided by Eschrich. As with macroscopic analysis, microscopy is not applicable to TTO authentication.

**8. Organoleptic Identification**

Prior to the development of modern chemical analysis, the assessment of aroma was the primary means to authenticate the essential oils. The odor evaluation is still part of most routine tests in quality control laboratories. The odor of tea tree oil is described as myristic in the WHO monograph. It is also characterized as having a spicy, fresh and camphor-like aroma with a dry hay-like undertone. While experts in organoleptic assessment of tea tree oil will be able to distinguish authentic TTO from other essential oils, and from the various TTO chemotypes, some of the subtler ways of adulteration may be missed. Therefore, the organoleptic evaluation is not suitable as a stand-alone method for TTO authentication, and has to be combined with an appropriate chemical method for an unambiguous determination of the identity.

**9. Genetic Identification and Distinction**

A few authors have looked into differences among nucleotide sequences of various gene regions for Melaleuca spp. and closely related species to determine phylogenetic relationships. Ladiges et al. and Brown et al. used the nuclear ribosomal 5S and internal transcribed spacer (ITS) regions to distinguish among Melaleuca, Callistemon, and related genera, but did not include any M. alternifolia samples.
Table 1. Scientific Names, Family, and Common Names of Known Tea Tree Oil Adulterants.*

<table>
<thead>
<tr>
<th>Speciesa</th>
<th>Synonym(s)a</th>
<th>Family</th>
<th>Common nameb</th>
<th>Other common namesc</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pinus roxburghii</em> Sarg.</td>
<td></td>
<td>Pinaceae</td>
<td>Chir pine</td>
<td>Long-leaf Indian pine</td>
</tr>
</tbody>
</table>

*aThe Plant List and the Kew Medicinal Plant Names Services database.1415 A comprehensive list of synonyms can be accessed through both websites.

*bHerbs of Commerce, 2nd ed.2

*cHerbs of Commerce, 2nd ed.,2 and the USDA GRIN database.16

* Note: The list of known adulterants is based on published data, e.g., those listed in the Botanical Adulterants Bulletin on TTO. The adulterating materials may not be the essential oil of the species listed in Table 1, but materials enriched in desirable terpenes obtained from the waste stream after rectification of camphor, eucalyptus, and pine essential oils. Materials obtained by fractional distillation from species not listed in Table 1 or pure compounds made by chemical synthesis, e.g., terpinen-4-ol or α-terpineol, may also be used to dilute TTO without the knowledge of the buyer.
essential oils are generally devoid of DNA. This is due to the authenticity of essential oils is not appropriate because leaves or twigs. The use of genetic technologies to determine materials such as authentication of crude genetic techniques is considered to be a suitable means for materials by genetic means are lacking.

M. alternifolia species and species from closely related genera, but data may be suitable to distinguish among the various harvesting, but also because unselected wild material yields lower amounts of oil overall.

1,8-cineole types. The manufacture of bush cut oil is not competitive with broad-acre production from tea tree cultivation, due to manual harvesting, but also because unselected wild material yields lower amounts of oil overall.

† Tea Tree oil was traditionally obtained from bush cuts, where leaf and twig are removed manually by machete from wild stands. Bush cut oil contains between 36-71% of 1,8-cineole, 6-22% of terpinen-4-ol, and 12-14% of α-pinene. Contents of 1,8-cineole, terpinolene, and terpinen-4-ol vary between 17-34%, 10-57%, and 1-20%, respectively, in the terpinolene chemotype.

11.1 Chemistry of Melaleuca alternifolia, Melaleuca linariifolia, Melaleuca dissita-flora and potential adulterants

Melaleuca alternifolia: The main compounds in tea tree oil are mono- and sesquiterpenes. Most often, authors have suggested three distinct chemotypes of M. alternifolia oil, dominated by 1,8-cineole, terpinolene, or (+)/(−)-terpinen-4-ol, respectively, although classification of up to seven chemotypes has been proposed. The TTO on the market is made from plants of the terpinen-4-ol chemotype. Besides 30-48% terpinen-4-ol, the oil of this chemotype contains 10-28% γ-terpinene, 5-13% α-terpinene, <0.01%-15% of 1,8-cineole, 0.5-8% p-cymene, 1-6% (+)/(−)-α-pinene, and 1.5-5% of terpinolene. Minor compounds include aromadendrene, δ-cadinene, ledene, limonene, and sabine.

The 1,8-cineole type M. alternifolia oil contains between 36-71% of 1,8-cineole, 6-22% of terpinen-4-ol, and 12-14% of α-pinene. Contents of 1,8-cineole, terpinolene, and terpinen-4-ol vary between 17-34%, 10-57%, and 1-20%, respectively, in the terpinolene chemotype. Of particular interest for the authentication of TTO are the enantiomeric ratios of (+)-terpinen-4-ol and (-)-terpinen-4-ol, as well as (+)-α-terpinol and (-)-α-terpinol. Ratios ranged between 63.3-69.8/36.7-30.2 for (+)-terpinen-4-ol/(−)-terpinen-4-ol and between 74.2-79.5/25.8-20.5 for (+)-α-terpinol/(-)-α-terpinol in authentic TTO. A phytochemical screening suggests that flavonoids, triterpenes, and tannins are also present in tea tree leaves. An ellagic acid derivative, 3,3′-di-O-methyllellagic acid-4,4′-O-glucoside, has been reported by Shah et al., but discrepancies among the NMR data, the alleged structure, and the structure drawing (3,5,3′,5′-tetrahydroxy-4,4′-dimethoxydiphenic acid-5-O-xylolpyranoside) cast a doubt about the veracity of these findings.

Melaleuca linariifolia: There are two main chemotypes described based on differences in the composition of essential oil obtained from the leaves and branchlets of this species: the 1,8-cineole and the terpinen-4-ol chemotypes. The oil of the terpinen-4-ol type is very similar to essential oils of M. alternifolia oil can be distinguished from M. alternifolia oil by its higher concentrations of t-sabinene hydrate and by

Figure 1: Major monoterpenes in tea tree oil

Note: The absolute configuration is often not indicated in the published literature. In these cases, the compounds are collectively referred to as terpinen-4-ol, α-pinene, and α-terpineol, respectively.

Edwards et al. used the chloroplast gene region NADH dehydrogenase F (ndbF) in addition to morphologic criteria to establish a phylogenetic relationship within the Melaleucaeae tribe, and found that the ndbF region is better resolved than the ITS region. As such, the ndbF genetic region may be suitable to distinguish among the various Melaleuca species and species from closely related genera, but data on successful authentication of commercial M. alternifolia materials by genetic means are lacking.

Comments: As outlined by the studies above, the use of genetic techniques is considered to be a suitable means for authentication of crude M. alternifolia materials such as leaves or twigs. The use of genetic technologies to determine the authenticity of essential oils is not appropriate because essential oils are generally devoid of DNA. This is due to the lack of solubility of DNA in highly lipophilic materials such as essential oils, and the low volatility of DNA (since the production of these oils using steam-distillation, DNA would have to be volatile to be present in the essential oil).

10. Physicochemical Tests

Several monographs include specifications for the density, optical rotation, refractive index, and/or miscibility of TTO in ethanol. While these simple tests are helpful as a screening test for TTO adulteration, they must be used in combination with a chemical analysis to rule out adulteration with some of the materials mentioned in section 5.

11. Chemical Identification and Distinction

A large number of analytical methods has been published for identifying TTO based on its chemistry. These methods are cited in the Laboratory Methods section below (Section 11.2). Distinction based on the phytochemical profile requires detailed knowledge of the constituents of TTO, its chemotypes, and its adulterants. The important components in TTO and its adulterating species are listed below. When distinction is based on chromatographic or spectral patterns, identification of specific constituents may not be necessary.

1 Tea Tree oil was traditionally obtained from bush cuts, where leaf and twig are removed manually by machete from wild stands. Bush cut oil is typically from older leaf which naturally has higher p-cymene (as high as 10%) contents, and variable amounts (sometimes below 35%) of terpinen-4-ol. It is also collected from a more genetically diverse mixture of plants, and so it may contain more material from the higher 1,8-cineole types. The manufacture of bush cut oil is not competitive with broad-acre production from tea tree cultivation, due to manual harvesting, but also because unselected wild material yields lower amounts of oil overall.
the ratio of α-pinene to α-thujene.\textsuperscript{35} No information on compounds other than the essential oil could be retrieved.

*Melaleuca dissitiflora:* Essential oil of the terpinen-4-ol chemotype of *M. dissitiflora* is also accepted as “tea tree oil” by the European Pharmacopoeia, and was listed in older ISO standards as a source of TTO.\textsuperscript{26,36} However, the most recent ISO standard does not include *M. dissitiflora* as an acceptable source, partly because the species is not important in commerce.\textsuperscript{9} There are two chemotypes of *M. dissitiflora*, distinguished by the concentrations of 1,8-cineole (63-66% and 2-7%, respectively). The 1,8-cineole chemotype also contains 5-7% limonene, 1-2% of α-pinene, 3-4% of terpinolene, and 1-7% of terpinen-4-ol.\textsuperscript{37} Williams and Lusunzi later analyzed the leaf oils of 30 *M. dissitiflora* trees from the Alice Springs region, which were mainly of the terpinen-4-ol (low 1,8-cineole) type but noticed a few oils with intermediate levels of 1,8-cineole.\textsuperscript{38} The composition of the oil made from the terpinen-4-ol chemotype is similar to TTO of *M. alternifolia*, but has generally higher (0.7-7.6%) sabinene concentrations.\textsuperscript{39} A recent publication suggested that methyl eugenol\textsuperscript{‡} levels are also substantially higher (0.7-7.6%) in *M. Alternifolia* than in *M. alternifolia* or *M. linariifolia*. The data were limited to one *M. dissitiflora* product, though, and need to be confirmed with a larger sample size.\textsuperscript{40}

*Cinnamomum camphora:* The essential oil obtained from the wood, leaves, or twigs of the camphor tree shows substantial differences in the composition depending on the subspecies, varieties, chemotype, and plant part.\textsuperscript{41,42} As an example to illustrate the point, Zhu et al. analyzed three chemotypes from China, with chemotype I containing 50.0% of 1,8-cineole, 14.4% of α-terpinolol, 6.9% β-pinene, 3.1% bornyl acetate, and 0.3% camphor; chemotype II is made up of 81.8% borneol, 3.0% camphor, 2.8% of α-pinene, and 1.6% of 1,8-cineole; chemotype III contains 57.7% isoneralidol, 3.6% of α-terpinolol, 2.3% linalool, and 0.3% camphor.\textsuperscript{42}

The crude essential oils obtained for commercial use are rich in crystalline camphor, which is obtained in pure form after filter pressing.\textsuperscript{42} The remaining essential oil is rectified by fractional distillation, yielding a camphor-rich fraction, and several fractions low in camphor. The fraction with the lowest boiling point is known as white camphor oil, while higher boiling fractions are separated into brown (sometimes also termed yellow, red, or black) camphor oil depending on the safrole content providing its color) and blue camphor oil, the latter containing mainly sesquiterpenes.\textsuperscript{43} White camphor oil, which is the product that is described as a TTO adulterant, contains mainly monoterpenes, e.g., 1,8-cineole, α-pinene, α-terpinolol, camphor, camphene, furfural, limonene, β-pinene, and safrole.\textsuperscript{44,45} Lumpkin et al. suggest that α-terpinene and sabinene are also among the main compounds in white camphor oil.\textsuperscript{46} Quantitative data report the contents of 1,8-cineole, α-pinene, and camphor at 46%, 22% and 21%, respectively.\textsuperscript{47} Unpublished results from close to 100 samples of white camphor oil give the following ranges of the eight major compounds: 30-40% of 1,8-cineole, 14-30% limonene, 2.3-14.4% of α-pinene, 4.2-10.0% p-cymene, 0.5-9.0% of γ-terpinolol, 1.3-8.4% sabinene, 1.8-7.5% myrcene, and 0.9-5.2% β-pinene. In all these samples, the camphor level was below 1.5%. (E. Schmidt [University of Vienna] email communication, December 9, 2017) The high contents of 1,8-cineole and α-pinene can be used to distinguish white camphor oil from TTO.

*Eucalyptus globulus:* Among the numerous *Eucalyptus* spp., *E. globulus* is the main source of eucalyptus oil, since it is grown for the wood and pulp industry and the leaves are used for the essential oil production.\textsuperscript{48} The leaf contains 1.2-3.0% essential oil, with 65-80% of 1,8-cineole as the main component, and α-pinene, α-terpinolol, aromadendrene, β-pinene, globulol, limonene, and t-pinocarveol as minor constituents.\textsuperscript{49,51} Commercial eucalyptus oils are most often rectified. In the rectification process, the crude essential oil is treated with an alkaline substance and subjected to fractional distillation to remove a majority of mono- and sesquiterpenes, leading to a product

\(‡\) In the European Union, the permissible levels of methyl eugenol from natural sources have been restricted to 0.001% in rinse-off products (e.g., shower gels, bar soaps) and to 0.0002% in leave-on (creams, lotions) and oral hygiene products.
that contains higher amounts in 1,8-cineole.\(^6\) In order to comply with the European Pharmacopoeia monograph on eucalyptus oil, the content of 1,8-cineole has to be greater than 70%, with other components in the following ranges: α-pinene: 0.05-10.0%; β-pinene: 0.05-1.5%; sabinene: not more than 0.3%; α-phellandrene: 0.05-1.5%; limonene: 0.05-15.0%; camphor: not more than 0.1%.\(^52\) The US National Formulary (NF) standard requires the oil to contain not less than 70.0% and not more than 95.0% of 1,8-cineole.\(^53\) The standard published by the International Organization for Standardization (ISO 3065:2011) demands an even higher content of 80-85% of 1,8-cineole.\(^54\) Besides the essential oil, eucalyptus leaves contain ellagitannins, proanthocyanidins, flavonoids, triterpenes, and formylated phloroglucinol derivatives.\(^50\) The latter are characteristic for the genus Eucalyptus. Besides E. globulus, the European Pharmacopoeia, the National Formulary, as well as the Personal Care Products Council’s International Nomenclature of Cosmetic Ingredients allows essential oils made from the leaves of E. polybractea and E. smithii to be sold as eucalyptus oils, as long as these oils comply with the composition outlined in the monograph.\(^52\) Similarly, the ISO standard allows E. radiata ssp. radiata, E. smitii, E. plenisisma, E. dives and other 1,8-cineole-rich eucalyptus species as sources of eucalyptus oil.\(^54\) Chemically, the large amount of 1,8-cineole can be used to distinguish eucalyptus oil from TTO.

It is not clear to what extent commercial eucalyptus oil is used to adulterate TTO, but reports suggest that the waste oil products obtained during the rectification process may represent a more important source of adulterants. Publications detailing the composition of these waste oils could not be retrieved, but since fractional distillation is used for rectification, some of the compounds found in TTO (e.g., α-pinene, α-terpineol, limonene) may be obtained in high amounts among the purified fractions and used to dilute authentic TTO.

**Melaleuca cajuputi**: There are three morphologically distinct subspecies, M. cajuputi subsp. cajuputi, subsp. cumingiana, and subsp. platyphylla. The chemical composition of essential oils derived from each of the subspecies is markedly different. Cajuput oil, which can also be obtained from M. leucadendra (see below) is also a medicinally used oil, e.g., as an ingredient in topical products to treat sore muscles or as a topical decongestant. The commercial cajuput oil is primarily made using leaves and branchlets of the subspecies cajuputi, of which there exist various chemotypes based on the concentrations of 1,8-cineole, which is present between 3-60%.\(^39,55\) The sesquiterpene alcohols globulol (trace–9%), viridiflorol (trace–16%) and spathulenol (trace–30%) are present in rather variable concentrations. Minor compounds in cajuput oil are α-pinene, α-terpineol, limonene, β-caryophyllene, humulene, and viridiflorene.\(^55,56\) However, cajuput oils from Myanmar, Thailand, Vietnam, or Indonesia may have an altogether different composition.\(^57,59\) Overall, the essential oil of M. cajuputi can be distinguished from TTO by the larger relative concentration of 1,8-cineole, and lower levels of terpine-4-ol, γ-terpinene, and α-terpinene.\(^50\)

**Melaleuca leucadendra**: M. leucadendra oil has two distinct chemotypes; chemotype I contains 10-45% Table 2. Comparison among the different chemical methods to authenticate tea tree oil

<table>
<thead>
<tr>
<th>Method</th>
<th>Pro</th>
<th>Contra</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPTLC</td>
<td>Quick</td>
<td>No statistics</td>
</tr>
<tr>
<td></td>
<td>Basic systems affordable</td>
<td>High-end equipment expensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dilution with essential oil fractions from other materials may be difficult to detect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Need for standard compounds</td>
</tr>
<tr>
<td>GC-FID</td>
<td>Standard equipment in many laboratories</td>
<td>Mainly quantitative method</td>
</tr>
<tr>
<td></td>
<td>Basic systems affordable</td>
<td>Dilution with essential oil fractions from other materials difficult to detect</td>
</tr>
<tr>
<td></td>
<td>Detection of adulteration possible using a fingerprint and concentration ranges</td>
<td>Need for standard compounds</td>
</tr>
<tr>
<td>GC-FID (chiral)</td>
<td>Detection of adulteration possible based on enantiomeric ratios of (+)/(-)-terpinen-4-ol and (+)/(-)-α-pinene</td>
<td>Mainly quantitative method</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Higher costs compared to conventional GC-FID</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Need for standard compounds</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Qualitative and quantitative State-of-the-art statistical evaluation possible</td>
<td>Equipment expensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dilution with essential oil fractions from other materials difficult to detect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Need for standard compounds</td>
</tr>
<tr>
<td>MIR/NIR</td>
<td>Quick</td>
<td>Mostly qualitative</td>
</tr>
<tr>
<td></td>
<td>Affordable</td>
<td>Accuracy and precision for low-concentration compounds insufficient</td>
</tr>
<tr>
<td></td>
<td>State-of-the-art statistical evaluation possible</td>
<td>Dilution with essential oil fractions from other materials difficult to detect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Need to build-up reference library</td>
</tr>
</tbody>
</table>
of 1,8-cineole as the main component, and 5-22% of \( \beta \)-cymene, 4-19% of \( \alpha \)-pinene, 3-6% limonene, and 6-9% of \( \alpha \)-terpineol.\(^{37,61}\) Chemotype II, also called the aromatic ether chemotype, is dominated by methyl eugenol (95-97%) or methyl isoeugenol (74-88%). Minor terpenes in the oil of chemotype II include \( \epsilon \)-\( \beta \)-ocimene, and calamene.\(^{39}\) Chemotype I is distinguished from TTO by the higher contents of 1,8-cineole, while chemotype II differs by the large amounts of methyl eugenol or methyl isoeugenol.\(^{37}\)

**Melaleuca quinquenervia:** According to *Hager’s Handbuch der Pharmazeutischen Praxis*, the essential oil of broadleaf paperbark (also called cajuput oil or niaouli oil depending on the author, although the latter should be derived from *M. viridiflora*)\(^{2,39}\) from Madagascar can be separated into four chemotypes: chemotype I has been reported to contain 37% of 1,8-cineole, 24% viridiflorol, 9.3% of 8, 9.3% of viridiflorene, 5.8% of 7 and 5.8% of \( \alpha \)-thujene. Chemotype II contains 22.8% of 1,8-cineole, 20% viridiflorol, 4.8% of \( \alpha \)-pinene, and 4.8% \( \alpha \)-thujene. Chemotype III is characterized by high amounts (47.8%) of viridiflorol, followed by \( \beta \)-caryophyllene (8.5%), 1,8-cineole (8.2%), and ledol (4.4%). Finally, chemotype IV consists predominately of E-nerolidiol (86.7%), with lesser amounts of \( \beta \)-caryophyllene (3.5%), and 1,8-cineole (1.1%).\(^{39}\) Two additional chemotypes have been described from Australia and Papua New Guinea. Chemotype V, commonly known as Nerolina, is comprised of \( \beta \)-nerolidol (74–95%) and linalool (14–30%) and is found along the east coast of Australia. Chemotype VI contains predominantly 1,8-cineole (10–75%) or viridiflorol (13–66%), with \( \alpha \)-terpineol (0.5–14%) and \( \beta \)-caryophyllene (0.5–28%) occurring at lower concentrations. Trees yielding essential oil of this chemotype are found from Sydney along the eastern coast of Australia and north to Papua New Guinea and New Caledonia.\(^{62}\) *Melaleuca quinquenervia* oil can be distinguished from TTO by high amounts of either 1,8-cineole, viridiflorol, or \( E \)-nerolidol.

**Pinus spp:** A number of pine species are used for essential oil production.\(^{63}\) Pine oil can be obtained by steam distillation of the needles, young shoots, and young branches with shoots and needles (pine needle oil) or of the wood chips of the heartwood and roots (pine oil). Important sources of pine needle oil are *P. mugo*, *P. palustris*, and *P. sylvestris*.\(^{64,65}\) While a number of pine species are used to produce essential oil from the wood, the largest volumes of turpentine oil are obtained from *P. masoniana*, *P. pinaster*, and *P. roxburghii*.\(^{66}\) (E. Schmidt [University of Vienna] email communication, December 9, 2017)
Due to the high cost of pine needle oil, only the use of pine resin oil makes economic sense as an adulterant of TTO. Many turpentine oils can be used as adulterants, primarily as a source of α-pinene and β-pinene. These two compounds may be used directly to dilute TTO or may serve as starting materials for the semi-synthesis of additional monoterpenes.

The turpentine oil of Masson pine is dominated by α-pinene (84.6%) and β-pinene (9.6%), with lower concentrations of limonene (1.7%) and longifolene (0.4%). Maritime pine contains 63-65% of (-)-α-pinene, 18-27% (-)-β-pinene, ca. 8% limonene, and traces of terpinene, camphene, and myrcene, while the turpentine oil from Chir pine is dominated by 3-carene, which makes it a less likely source as adulterant.

Tea tree oil and pine oils can be differentiated by the altogether different composition, with pine resin oils dominated by α-pinene and/or β-pinene, or 3-carene, while TTO contains substantially higher amounts of terpinen-4-ol, γ-terpinene and α-terpinene.

11.2 Laboratory methods

Note: Unless otherwise noted, all methods summarized below are based on chemical analysis of the essential oil. Analytical tests evaluating tea tree leaf extracts are beyond the scope of this document.

11.2.1 HPTLC

Methods from the following sources were evaluated in this review: the European Pharmacopoeia (EP 7.0), the British Pharmacopoeia (BP), and the High-Performance Thin Layer Chromatography (HPTLC) Association. Comments: The conditions described in the EP and BP are the same, and differ from those described by the HPTLC Association in that the EP/BP method uses a mobile phase of higher polarity and lists 1,8-cineole, terpinen-4-ol, and α-terpineol as reference compounds rather than the two compounds (1,8-cineole and nerolidol) suggested by the HPTLC Association. Both methods detail appropriate conditions to separate the essential oil constituents of TTO. Developed to detect adulteration of niaouli (Melaleuca viridiflora) essential oil, the HPTLC Association’s method provides suitable separation for M. alternifolia leaf oil, and enables its distinction from cajuput oil, eucalyptus oil, kanuka (Kunzea ericoides, Myrtaceae) oil, manuka (Leptospermum scoparium, Myrtaceae) oil, and neroli (Citrus aurantium var. amara, Rutaceae).

Since the method proposed by the HPTLC Association (Figure 2) has documented its suitability to detect adulteration with a variety of potential TTO adulterants based on an evaluation of the fingerprints, it is suitable for the routine identity testing in a quality control laboratory. However, dilution of tea tree oil with essential oil fractions

Figure 2: HPTLC analysis of commercial tea tree and authentic Melaleuca spp. essential oils

Lane 1: Isoeugenol and isoeugenyl acetate (with increasing Rf); Lane 2: α-terpineol, terpinen-4-ol, and 1,8-cineole (with increasing Rf); Lanes 3-9: commercial tea tree oils; Lanes 10-12: Melaleuca alternifolia oils; Lanes 13-15: Melaleuca linariifolia oils; Lanes 14-16: Melaleuca dissitiflora oils. Conditions as specified by the HPTLC Association. Detection after derivatization with anisaldehyde reagent. Top: white light; bottom: UV at 366 nm. Image provided by Camag AG; Switzerland.
obtained from the waste stream of a number of essential oils may be difficult to detect, and may warrant the quantitative determination of the individual tea tree oil compounds and the enantiomeric ratios of terpinen-4-ol and α-terpineol.

### 11.2.2 Infrared, mid-infrared, and near infrared spectroscopy

Four infrared-based authentication methods were evaluated in this review (Tankeu et al., Gallart-Mateu et al.):

**Comments:** Sixty-four TTO samples were evaluated using near-infrared (NIR), or mid-infrared (MIR), and compared to results obtained by gas-chromatographic (GC) methods. The concentrations of seven major essential oil components (1,8-cineole, terpinolene, terpinen-4-ol, γ-terpinene, α-terpinene, α-terpineol, and limonene) were calculated using a partial least square regression analysis based on quantitative GC data. Based on the published results, the quantitative models constructed for the infrared data provides a fairly good correlation with the results from the GC measurements. Partial least square (PLS) regression models were constructed based on MIR versus GC-MS and NIR versus GC-MS results, with coefficients of determination ranging from 0.76 – 0.97 for MIR, and 0.75 - 0.95 for NIR. In general, the coefficients of determination were higher for the models constructed with MIR data compared with NIR data. Prediction of quantitative levels was better with compounds at higher concentrations than those, e.g., limonene, that are in the low single percentage range. In the second study, a set of 267 samples was used to build a chemometric model for FT-IR and NIR (Figures 3 and 4) authentication of tea tree oil. The models were built using a partial least square analysis. After optimization of the models, the overall accuracy of FT-IR was 87% (153 correctly assigned samples out of 175 that were tested by FTIR), and 98% for NIR (3 misidentified samples out of 125 analyzed by NIR).

The FT-IR, MIR and NIR methods provide a fast, easy and affordable approach to get a good idea about the TTO quality. Unusual amounts of any of the seven target compounds can be used to detect adulteration, and the chemometric model based on the NIR method by Gallart-Mateu et al. provided good accuracy. While highly sophisticated types of adulteration may be difficult to detect with this method, it has a lot of promise as a method in routine quality control laboratories. Samples that are close to the discriminant threshold (the limit that separates the authentic from the adulterated samples) in this NIR model can be verified using a GC method (see section 11.2.3). For its use in quality control, further method validation is needed, and system suitability parameters must be established.

### 11.2.3 Gas chromatography

Methods described in the following literature were evaluated in this review: EP 7.0, ISO 4730:2017, Leach et al., Brophy et al., Gallart-Mateu et al., Wong et al., Wang et al., Sciarrone et al., Shellie et al.

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Figure 3: Fourier transform-infrared (FT-IR) spectra of tea tree oil samples in the range between 4,000 and 600 cm⁻¹

Image provided by Prof. Miguel de la Guardia (University of Valencia, Valencia, Spain)

Figure 4: Near infrared (NIR) spectra of tea tree oil samples in the range between 14,000 and 4,000 cm⁻¹

Image provided by Prof. Miguel de la Guardia (University of Valencia, Valencia, Spain)
Padalia et al., and Southwell et al. Specific comments on strengths and weaknesses of each of the methods are listed in Appendix 1, Table 3.

**Comments:** Gas chromatography (Figure 5) has been the method of choice to analyze TTO for decades. Sample preparation consists of a dilution of the analyte in acetone, ethanol, or hexane. For routine analysis, the validated methods published by the European Pharmacopoeia or ISO 4730:2017 represent attractive choices. Parameters for composition ranges of a number of TTO constituents have been tightened in the ISO 4730:2017 (versus the 2004 version) to better reflect the quality of TTO being produced currently, and to mitigate the trading of adulterated oils.

Adulteration of TTO can occur with addition of terpinen-4-ol, a molecule which can be readily synthesized. In evaluation of TTO quality, the enantiomeric ratio of terpinen-4-ol provides a clear indication of the authenticity. The enantiomeric ratio of chiral terpenoids is genetically predefined and serves as a useful signature for essential oil identification. The application of chiral GC methods to measure enantiomers of terpinen-4-ol helps to detect the addition of this compound from synthetic or natural non-TTO sources.

Multi-dimensional GC analysis is an interesting approach, in particular in a research setting, but is impractical for commercial use due to costs (combination of two columns) and time savings since acceptable peak separation can be achieved by conventional or chiral chromatography.

12. Conclusion

Identification of TTO can be achieved by a range of analytical techniques. In practice, gas chromatography combined with physical measurements of optical rotation, refractive index, density and miscibility in ethanol, provide a robust identification of TTO. While HPTLC and NIR represent good screening methods, more sophisticated types of adulteration will require the use of chiral GC to establish TTO authenticity with confidence.

13. References

5. Tea Tree Oil – In support of an iconic Australian industry. Barton, ACT, Australia: Rural Industries Research and Development Corporation (RIRDC); 2013:1-20.


Appendix 1

Table 3: Comments on the published GC methods for tea tree oil. In order to compare methods, the run times indicate only the duration of gradient elution, since initial and final hold times are often not indicated.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>EP 7.026</td>
<td>This is a validated GC-FID method with 36-minute run time using a stationary phase similar to the polar column used in the ISO standard 4730:2017. Authentication is based on the ranges of 11 TTO components. The determination of enantiomeric ratios for authentication of TTO oil is not part of the EP monograph.</td>
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<tr>
<td>ISO 4730:2017</td>
<td>This ISO standard includes three different methods using columns of low, medium and high polarity and run times of 65 min., 20 min., and 30 min., respectively. Depending on the conditions, the peaks of 1,8-cineole, β-phenelendrene, or α-cymene are not resolved. The determination of the terpinen-4-ol enantiomer ratio is proposed as additional measure to ensure authenticity, but no specific conditions to measure this ratio are given.</td>
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<tr>
<td>Leach et al.72</td>
<td>This GC-MS method separates the TTO constituents in 43 minutes. The use of a chiral column allows the determination of terpinen-4-ol, α-pinene, and α-terpineol enantiomers. The resolution among the peaks is acceptable, despite the fact that terpinen-4-ol enantiomers are not fully resolved. Suitable to detect adulteration of TTOs; method validation data are lacking.</td>
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<tr>
<td>Brophy et al.73</td>
<td>The publication details two routine GC methods using FID detection. The stationary phases are of low and high polarity with run times of 21 min. and 13 min., respectively (not including the hold time at the end, which was not detailed in the paper). The peaks of 1,8-cineole, β-phenelendrene, and limonene are not always resolved, which hampers accurate quantification of 1,8-cineole, one of the markers of adulteration. The method is able to detect low quality or adulterated TTOs based on low concentrations of terpinen-4-ol and high contents of 1,8-cineole. Method validation data are lacking.</td>
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<tr>
<td>Gallart-Mateu et al.74</td>
<td>This validated GC-MS method is modified from Brophy et al.73 using a low polarity column with a run time of 17 min. Limonene and 1,8-cineole are not resolved, but the use of selective ion monitoring may allow quantification of these compounds. The use of a headspace injector reduces the amount of solvent necessary for the sample preparation. Data on the method's ability to detect adulteration are not presented, but it is expected to give results similar to other GC-MS fingerprinting methods.</td>
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<tr>
<td>Sciarrone et al.78</td>
<td>Sciarrone et al. assessed the quality of TTO using a chiral GC column with a FID detector, and bi-dimensional GC-MS (using a heart-cut system) combining a non-polar and polar stationary phase with FID and MS detection. The run times for each of the separations are long at 75-77 min. The use of a chiral separation allows determination of the enantiomeric ratios of (+)-terpinen-4-ol, (-)-terpinen-4-ol, (+)-α-pinene, (-)-α-pinene, (+)-α-terpineol, (-)-α-terpineol, and (+)-limonene/(-)-limonene as indicators of tea tree oil authenticity without the need of a costly MS instrument. However, it is not clear how widespread instrumentation that allows running bi-dimensional GC is in the industry. The method has not been validated.</td>
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<tr>
<td>Shellie et al.79</td>
<td>This method represents another bi-dimensional GC approach using two columns of different polarity. The compounds elute directly from column 1 onto column 2 after being trapped using a cryogenic modulator. Due to the improved resolution, this seems a suitable approach for the detection of adulteration, although actual data on the determination of TTO identity are not presented. With run times of 75 minutes in each dimension, this method is too time-consuming for a routine assay. The method has not been validated.</td>
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<tr>
<td>Padalia et al.80</td>
<td>Padalia et al. describe two methods using either FID or MS detection on columns of low polarity with a 60 min. run time for each method. The peaks of 1,8-cineole, p-cymene, and limonene are not resolved, which prevents accurate quantification of 1,8-cineole. No data on its ability to detect adulteration are given, but due to the similarity with other methods, the GC-FID or GC-MS fingerprints might be used as a criterion for authentication of TTO. Method validation data are not presented.</td>
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<tr>
<td>Southwell et al.81</td>
<td>Two different GC-FID methods are described in this paper. One method, using an intermediate polarity column, allows distinguishing M. alternifolia and M. linearifolia chemotypes in 21 min. based on concentrations of 1,8-cineole, terpinolene, and terpinen-4-ol. Enantiomeric GC analysis enables verification of the ratios of (+)-terpinen-4-ol/(-)-terpinen-4-ol, (+)-α-pinene/(-)-α-pinene, (+)-α-terpineol/(-)-α-terpineol, and (+)-limonene/(-)-limonene. The run time for the chiral separation is 85 min. The authors point out that the chiral separation becomes overly labor intensive if ethanol extracts of tea tree leaves are used rather than the distilled oil due to the need for increased column cleaning. While the combination of these two methods provide adequate data to determine the authenticity of TTO, the 85-minute chiral separation may prove too long for adoption in a quality control setting. Method validation data are not given.</td>
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