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Evaluation of fast enantioselective multidimensional gas chromatography methods for monoterpenic compounds: Authenticity control of Australian tea tree oil

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ABSTRACT

This work demonstrates the potential of fast multiple heart-cut enantioselective multidimensional gas chromatography (GC – *e*GC) and enantioselective comprehensive two-dimensional gas chromatography (*e*GC × GC), to perform the stereoisomeric analysis of three key chiral monoterpenes (limonene, terpinen-4-ol and α-terpineol) present in tea tree oil (TTO). In GC – *e*GC, separation was conducted using a combination of mid-polar first dimension (^{1}D) column and a chiral second dimension (^{2}D) column, providing interference-free enantioresolution of the individual antipodes of each optically active component. A combination of ^{1}D chiral column and ^{2}D polar columns (ionic liquid and wax phases) were tested for the *e*GC × GC study. Quantification was proposed based on summation of two major modulated peaks for each antipode, displaying comparable results with those derived from GC – *e*GC. Fast chiral separations were achieved within 25 min for GC – *e*GC and < 20 min for *e*GC × GC, while ensuring adequate interference-free enantiomer separation. The suitability of using these two enantioselective multidimensional approaches for the routine assessment of chiral monoterpenes in TTO was evaluated and discussed. Exact enantiomeric composition of chiral markers for authentic TTOs was proposed by analysing a representative number of pure TTOs sourced directly from plantations of known provenance in Australia. Consistent enantiomeric fractions of 61.6 ± 1.5% (+):38.4 ± 1.5% (−) for limonene, 61.7 ± 1.6% (+):38.3 ± 1.6% (−) for terpinen-4-ol and 79.6 ± 1.4% (+):20.4 ± 1.4% (−) for α-terpineol were obtained for the 57 authentic Australian TTOs. The results were compared (using principle component analysis) with commercial TTOs (declared as derived from *Melaleuca alternifolia*) obtained from different continents. Assessing these data to determine adulteration, or additives that affect the enantiomeric ratios, in commercially sourced TTOs is discussed. The proposed method offers distinct advantages over *e*GC, especially in terms of analysis times and selectivity which can serve as a reliable platform for authenticity control of TTO.

1. Introduction

Melaleuca (family Myrtaceae), an Australian native plant, represented by more than 280 species with 37 infraspecific taxa, has a natural distribution almost entirely in different regions of Australia [1]. Among the various species, *Melaleuca alternifolia* is notable for its high commercial value, where the essential oil found in its foliage has been valued for pharmaceutical, perfumery and industrial uses. The extracted volatile oil, commonly referred to as tea tree oil (TTO), has been reported to possess a wide spectrum of biological activity including anti-fungal, anti-viral, anti-protozoal, anti-microbial, anti-inflammatory, and anti-cancer properties [2–4]. Today, TTO is widely traded in international markets which span the globe. Brophy et al. report that total annual world production of TTO is in excess of 600 t, with Australia being the world biggest producer/exporter [1].

TTO consists mainly of terpenoids, particularly monoterpenes and sesquiterpenes, and their associated oxygenated analogues. Generally, the chemical composition of TTO traded internationally is regulated by an international standard (ISO 4730:2004) which specifies levels for 15 compounds found within the oil as well as its physical properties [5]. Lately, strong global demand for TTO has increased the incidence of adulteration in TTO; supply of pure TTO has not been able to keep up with demand [6]. Recent reports

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highlighted concern that pure Australian TTO is being intentionally adulterated, extended or otherwise modified with undisclosed lower-cost ingredients [6]. This is not surprising as incidents of adulteration in essential oils have been frequently reported, involving mostly substitution or amendment with synthetic or natural compounds, or a cheaper essential oil [7]. Currently, there are no official protocols available to precisely determine chemical authenticity of TTO, since available international standards generally focus on bulk physical measurement, and no detailed chemical analysis apart from abundances of certain compounds. Classical measurement of optical rotation lacks selectivity for mixture analysis, where addition of synthetic or other sources of monoterpenes (either dextrorotary or levorotary) can be used to adjust the optical rotation to closely approximate the desired range to meet relevant standard criteria. Therefore, it is of importance to develop suitable authenticity assessment protocols for TTO in view of their growing trend towards adulteration.

Chiral analysis of selected isomeric volatiles (normally terpenic compounds) is one of the available alternatives to define origin or possible adulteration of an essential oil product. Currently, enantioselective gas chromatography (*e*GC) is the most widely used technique for enantiomeric separation of chiral volatile organic compounds. Several *e*GC methods have been recently reported for the separation of a range of terpenic compounds in TTO [8,9]. However, classical *e*GC methods may be incapable of distinguishing the target enantiomers from other interfering compounds in the sample matrix; non-specific co-elutions may arise. It should be noted that for accurate and reliable determination of enantiomeric fraction (*EF*), enantiomeric excess (*ee*) or enantiomeric ratio (*ER*), each peak of the chiral compound should be well resolved and comprise a single component.

In this respect, heart-cut multidimensional gas chromatography (GC–GC) is one of the available options to address non-specific overlap encountered in *e*GC by combining two or more separation steps. In an enantioselective GC–GC (GC–*e*GC) system, the classical approach is to use a chiral second dimension (^{2}D) column, where a single or a few selected regions (containing the target compounds) from the achiral first dimension (^{1}D) column are H/C to the chiral ^{2}D column to effect the chiral separations. This method has been exploited for enantioselective analysis of several monoterpenic compounds in TTO [10]. However, some major drawbacks frequently encountered in classical GC–*e*GC are lengthy analysis time (>90 min [10]) and lack of stability or repeatability which limit their use for routine quality control purposes. An advanced approach based on comprehensive two-dimensional gas chromatography (GC × GC) has also been reported for chiral analysis of TTO [11]. However, the requirement for skilled operators, lengthy analysis time and lack of validation criteria for practical operation has hindered the use of this technique for routine purposes.

Despite the number of reports on chiral analysis of TTO, few conclusive data are available concerning the 'true' enantiomeric composition of chiral terpene markers owing to the need for a methodology that offers highest selectivity for unbiased measurement of the chiral ratios, and to source a representative number of authentic samples. In the present work, we focus on: (i) development of fast and accurate GC–*e*GC and *e*GC × GC approaches for the stereoisomeric analysis of limonene, terpinen-4-ol and α-terpineol in TTO; (ii) examination of chromatographic approaches to achieve fast chiral separation by GC–*e*GC and *e*GC × GC; (iii) measurement of *EF*s of target monoterpenes for authentic Australian TTOs, and (iv) comparison of the respective ratios across different samples from a number of continents. The analytical practicability of the proposed method was further demonstrated in the enantio-analysis of a large number of samples (n = 102). This report represents the most complete and reliable study available for the enantioselective analysis of limonene, terpinen-4-ol and α-terpineol in TTO, representing a valuable database for determining authenticity of TTO.

2. Experimental

2.1. Chemicals and reagents

(+)- and (−)-limonene (>98% purity), α-pinene, 1,8-cineole, α-terpinene, γ-terpinene and p-cymene (≥98% purity) were provided by Australian Botanical Products (Hallam, Australia), and mixtures of terpinen-4-ol and α-terpineol were provided by FGB Natural Products Pty Ltd (Oakleigh South, Australia). Analytical reagent grade acetone was purchased from Merck (Darmstadt, Germany).

2.2. TTO samples

A total of 57 pure Australian TTO plantation samples (derived from *M. alternifolia*) of known provenance were collected by the Australian Tea Tree Oil Industry Association (ATTIA, Canberra, Australia) from five production areas around Queensland and New South Wales. All the collected samples (covering a harvest period of more than 6 y) were steam distilled from the foliage and terminal branches of the respective plant according to the standards and guidelines provided by ATTIA Code of Practice [12]. Yields ranged from ca. 1 to 2% of wet material weight; these oils conformed to ISO 4730:2004. Commercial TTO (45 samples) together with their respective chain of custodies were sampled from Australia (AUS), New Zealand (NZ), China (CN), United States of America (USA), United Kingdom (UK) and Germany (DE), and provided by ATTIA. Details of the region of Australia and country of purchase of samples are listed in Supplementary Information Table S1 and Table S2. Prior to analysis, samples were diluted using acetone to prepare 0.25% (v/v) volatile oil solutions.

2.3. Instrumentation

2.3.1. e*GC–flame ionisation detection (FID) system*

*e*GC–FID analyses were conducted on an Agilent Technologies 6850 Network GC system (Agilent Technologies, Mulgrave, Australia) equipped with an auto sampler (model 7683 series) and split/splitless inlet. The chromatographic separation was performed using an Astec CHIRALDEX B-PM (2,3,6-tri-O-methyl β-cyclodextrin) capillary column of dimensions 30 m × 0.25 mm I.D. × 0.12 μm film thickness (d_f) supplied by Sigma-Aldrich (Supelco; Bellefonte, PA); this chiral column was selected after a comparative column study (see below). Data acquisition was performed with Agilent ChemStation software (version Rev. B.04.03 [version 16]). The chromatographic conditions were: oven temperature program 50 °C to 80 °C at 3 °C min^{-1}, then to 105 °C at 10 °C min^{-1}, followed by 3 °C min^{-1} to 120 °C and 10 °C min^{-1} to 180 °C (hold 10 min); injector temperature 230 °C; carrier gas hydrogen at a constant flow rate of 1.5 mL min^{-1} (approximately 42 cm s^{-1}); injection volume 0.2 μL and using a split ratio of 100:1. The FID temperature was 250 °C.

2.3.2. GC–e*GC–MS system*

Separations were conducted on an Agilent 7890A GC system coupled with a 5975c quadrupole MS (MSD; Agilent Technologies). A microfluidic Deans switch device (DS; Agilent Technologies, part no. G2855B) for heart-cut (H/C) effluent switching was used to interface the end of the ^{1}D column to the start of the ^{2}D column with a deactivated fused-silica tubing (DFS; 1 m × 0.1 mm I.D.) as the transfer line to the FID. The H/C switching of effluent flow from the ^{1}D column to either the ^{2}D column or deactivated fused silica (DFS) to the FID was controlled through manipulation of the