



RURAL INDUSTRIES RESEARCH
& DEVELOPMENT CORPORATION

Authenticating

Essential Oil Flavours and Fragrances

Using Enantiomeric Composition Analysis

**A report for the Rural Industries Research
and Development Corporation**

by Professor RC Menary and Ms SM Garland
University of Tasmania

October 1999
RIRDC Publication No 99/125
RIRDC Project No UT-15A

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ISBN 0 642 57906 7
ISSN 1440-6845

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Published in October 1999
Printed on environmentally friendly paper by Canprint

Foreword

The introduction of international standards to quantify and qualify properties of essential oils has seen the increasing application of analytical technology. The food and flavour industry continues to move towards establishing standards which specify the preferred chemical profile of essential oils.

The enantiomeric ratio of optically active components of essential oils is often specific to the species and origin of the material used in the production of extracts and oils. It can be used to determine whether material of synthetic origin has been used to enhance the natural qualities of products, or if oils from other crops have been added to increase quantity or alter quality. Products may be rejected on the international market if the enantiomeric ratios of optically active components do not meet the standards expected for natural oils

The parameters of oils and extracts produced in Australia are considered to be specific to this region. This assumption needs to be tested scientifically. Without direct involvement in the standardisation process, Australia runs the risk of having to meet requirements established using reference material originating in different climates, from limited plant varieties and using different horticultural practices.

This project was established to determine the feasibility of establishing standard methods to characterise the enantiomeric compositions of the major essential oils produced in Australia.

This report, a new addition to RIRDC's diverse range of almost 400 research publications, forms part of our Essential Oils and Natural Plant extracts R&D Program which aims to support the growth of a profitable and sustainable essential oils and natural plant extracts industry in Australia.

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Managing Director

Rural Industries Research and Development Corporation

Acknowledgements

Thanks are extended to

Dr. Noel Davies
Central Science Laboratory
University of Tasmania

for his expertise in the operation of the GC MSD.

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Abbreviations

GC	Gas Chromatography
GCMS	Gas Chromatography Mass Spectrometry
NMR	Nuclear Magnetic Resonance
MSD	Mass Selective Detection
ee	Enantiomeric Excess
MDGC	Multi Dimensional Gas Chromatography
FID	Flame Photometric Detection
Rs	Resolution
SPME	Solid Phase Micro Extraction
SIM	Selective Ion Monitoring
MDC	Multi Dimensional Chromatography

Executive Summary

The quantification of components in oils has been used for quality assessment for many years. In a highly competitive market, the use of authenticated natural oils can provide an advantage. The adulteration of oils with synthetic derivatives and the bulking of extracts with oils from cheaper varieties is a method that may be used to undercut the price of natural extracts in world markets. Authenticating the origin and naturalness of oils can be done using an analytical technique which looks at the structural detail of the chemicals which make up the profile of an essential oil.

The orientation of atoms within a molecule is very specific. Molecules with exactly the same molecular formula, but with one atom orientated differently about a carbon atom, can have very different properties. These molecules are said to be chiral and each form is called an enantiomer. The lock and key mechanism of bioactive chemicals with biological receptors is so specific that only one enantiomeric form of a enantiomeric pair, will induce a response. When directed light is passed through a solution containing only one enantiomer form, the light will be rotated about it's axis, clockwise or anti-clockwise. It's enantiomeric opposite will rotate the light in the opposite direction. These chemicals are said to be optically active.

The biosynthetic pathway of the terpenes in essential oil crops often produce one form only of an optically active chemical. Sometimes one form of an enantiomer may be produced in larger amounts. Often the relative abundances of two enantiomeric form is very specific to the species and geographical origin of an essential oil and it can be used to authenticate oils. Technology, which allows chiroselective differentiation of optically active elements of essential oils, presents the possibility of establishing the authenticity and geographical origin of essential oils. This technology is now being used to introduce international standards to specify the preferred chemical profile of essential oils. Adulteration of natural products with synthetic substitutes or bulking oils from other crops can now be detected.

The suitability of chiral gas chromatography columns was assessed for their applicability to the resolution of enantiomers within oils important to the Australian industry. A review of the major essential oil products exported from Australia, identified lavender, lemon, boronia, peppermint, dill, eucalypt, tea tree and caraway oil as being some of the extracts of immediately relevancy to the industry. The chemical composition of these oils was reviewed. Components with a chiral centre were identified.

The use of enantiomeric ratios in distinguishing the geographical origin has been reported for many essential oils. Enantioselective columns have been used to elucidate biosynthetic pathways by correlating enantiomeric excesses of structurally related terpenes. Several limitations of the chiral assessment of essential oils were identified.

A great variety of well-defined authentic samples must be examined to provide a statistical background (Faber et al., 1997).

Chiral stability of many chiral chemicals in essential oils depends on the stage of fruit ripeness, pH value of plant material and technological influences during processing or storage of food stuffs. Racemates of natural origin can be generated by non-enzymatic reactions such as auto-oxidation and photo-oxidation.

Only chiral volatiles of high optical purity and with characteristic and small ranges of enantiomeric excesses can be used to validate the origin of essential oil components or detect the blending of natural and synthetic chiral flavour compounds.

Peak identity in the gas chromatographic analysis of chiral components, within the matrix of essential oils, is reliant on the availability of standards. The mass spectra of enantiomers are identical and there is a paucity of data on the relative retention times and Kovat indices of chiral chemicals on chiral columns.

Nevertheless, the systematic evaluation of origin specific enantiomers has proved to be a valuable criterion for differentiation natural flavour compound from those of synthetic origin.

The use of cyclodextrin derivatives for the stationary phase in enantioselective gas chromatography columns is now well established. Many types of chiral columns are now produced commercially. Papers reviewed, reported the use of a range of types, often using achiral columns in tandem with the chiral varieties. Multi-dimensional gas chromatography (MDGC) was not a feasible option within the time and budget constraints of this project. β -cyclodextrin chiral columns were selected for use in this study because of their broad applicability to many chemical types. A Hewlett Packard column was selected with a phenyl based polymer inter dispersed with β -cyclodextrin. Racemic mixes of enantiomer standards were prepared and tested.

Results indicated that 20% β -cyclodextrin columns on phenyl based polymers have wide applicability in essential oils, with baseline separation achieved for a great number of enantiomers investigated. Resolution was improved with slower temperature ramps. Not all chemicals investigated were separated on the chiral column. There was no obvious structural feature unique to these chemicals which could explain the lack of resolution. The solvent in which standards were injected did not affect the resolution.

SPME injection is suitable for the introduction of standards into the chiral column with no loss of resolution despite the lack of a cold trap to concentrate the volatiles at the head of the column. However, SPME is a manual injection and the loss of automation resulted in some variability in the retention times such that this parameter could not be used for peak identification. As such, SPME injection requires detection using mass spectra.

Separation of enantiomers of terpenes within the matrix of essential oils was achieved. Mass spectra was used for peak identification. Standards were required to confirm identification as there is little available data on the Kovat indices of chemicals specific to chiral columns. Where enantiomerically pure standards were not available, the absolute configuration of peaks separated could not be specified. Despite this, resolution was sufficient to allow for the comparison of enantiomeric ratios.

Oils from a range of geographical locations and from different clones were analysed to determine the effectiveness of the methods developed to identify enantiomeric excesses. Although insufficient samples were analysed to show whether patterns of enantiomeric ratios could be used to distinguish oils produced in different countries, it is evident that the techniques used are effective in establishing the profiles of the chiral components of oils.

Preliminary results indicated that boronia clones may be distinguished by the distribution of the enantiomers of some of the terpenes containing a chiral centre. Although a larger database is required for conclusive results, the Tasmanian clones 250 and 17 may be distinguished by the enantiomeric excesses of camphene.

Alternative columns should be investigated towards achieving resolution of optically active chemicals such as carvone and fenchone.

Over all, single dimension gas chromatography methods proved to be robust and reproducible. The obvious direction for further investigation within this field is the application of multi-dimensional gas chromatography (MDGC).

Introduction

The quantification of components in essential oils has been used for quality assessment for many years. Very little information about the origin or processing history of the oils, however, can be determined by the application of the standard techniques. Enantioselective biogenesis is fundamental to the functionality of many components of essential oils, with the bioactivity of many chemicals limited to only one stereochemical form. The enantiomeric ratio of optically active components of essential oils is often specific to the species and origin of the material used in the production of extracts and oils.

Technology, which allows chiroselective differentiation of optically active elements of essential oils, presents the possibility of establishing the authenticity and geographical origin of essential oils. This technology is now being used to introduce international standards to specify the preferred chemical profile of essential oils. Adulteration of natural products with synthetic substitutes or bulking oils from other crops can now be detected.

The characteristics of oils produced in Australia are often unique to this region. It is critical that our essential oil industry has sufficient scientific information to constructively contribute to the process of standardisation. It should not have to meet compositional parameters, which have been established, using reference material originating in different climates, from limited plant varieties and using different horticultural practices.

Objectives

1. To appraise the suitability of chiral gas chromatography columns for the elucidation of the enantiomeric composition of essential oils.
2. To undertake the separation of racemic mixes of standards using chiral columns.
3. To transfer the methodology to the analysis of oil and extract samples.

Methodology

Research methodology developed through 3 stages:

1. Assess the feasibility of measuring enantiomeric ratios in commercial essential oils using chiral columns.

A review of the major essential oil products exported from Australia, identified lavender, lemon, boronia, peppermint, dill, and caraway oil as being some of the extracts of immediately relevancy to the industry. The chemical composition of these oils was reviewed. Components with a chiral centre were identified.

A literature survey was conducted to determine the feasibility of separating and distinguishing optically active components of commercial essential oils using chiral columns.

In the field of essential oils, much of the characterisation uses GC MS with the retention times and mass spectra used to identify components by reference to databases. In the absence of reference data, determining the stereochemistry of components would normally require the isolation of each chemical. Nuclear magnetic resonance (NMR) would be required, amongst other analytical techniques.

With reference to previous studies, and the technology readily available, it was determined that the use of GC chiral columns was the most effective method to separate and quantify the enantiomeric composition of oils. However, the mass spectra of 2 enantiomer are identical and Kovat indices are unique to the column type used in the analyses. Confirmation of structure in this study required the direct comparison to known standards.

Enantiomerically pure standards were purchased from laboratory chemical suppliers where possible. Otherwise, components which had been isolated from natural essential oils, were used. In addition, retention times were determined by analysis of complete oils which naturally contain only one enantiomeric form of a chemical.

2. Undertake the separation of racemic mixtures using chiral columns

Standards were used to determine the retention times of enantiomers, with a range of conditions trialed to effect baseline resolution of racemic mixes where possible. Once separation was achieved the reliability of using retention time as confirmation of form was tested. MSD detection was employed to confirm the retention time of the enantiomers analysed.

3. Transfer methodology to the analysis of oil and extract samples.

Enantioselectivity has been used to prove the authenticity of flavours and fragrances. For that purpose a great variety of well-defined authentic samples must be examined to provide a statistical background. (Faber et al., 1997). Analyses of oils from a range of geographical locations and from different clones were analysed to determine the effectiveness of the methods developed to identify enantiomeric excesses.

Detailed Results

Chiral Chemicals in Essential Oils

Chiral centres exist in menthone, isomenthone, menthyl acetate, pulegone and menthol in the distilled oils of the *Mentha* species. Mint oils from Brazil and Japan were found to be nearly identical with respect to their enantiomeric distribution of (\pm)- α -pinene, (\pm)- β -pinene and (\pm)-limonene. However, all investigated *Mentha* species are differentiated with regard to their enantiomeric distribution of these 3 components (Mosandl, 1991). Mint oil of natural origin contains enantiomerically pure (-)-menthone, (+)-isomenthone, (-)-menthol and (-)-menthyl acetate.

Lavender oil can be characterised by determining the ee of linalyl oxides, linalool, linalyl acetate, borneol, bornyl acetate, α -terpineol and trans nerolidols. The optical purity of (R)-(-)-linalyl acetate is not influenced by the variety of lavender and it is also independent of workup and storage conditions (Mosandl, 1990). In diethyl-ether extracts and in steam distillates of lavender species, high optical purities of the (R)-(-)-linalyl acetate were found. Linalyl acetates can be used as an indicator of the naturalness of lavender. In comparison, increasing amounts of the (S)-(+)-linalool was detected in oils produced by hydro-distillation of longer than 1 hours duration (Kreis, 1992). The chiral stability of linalool is dependent on the stage of fruit ripeness, pH value of plant material and technological influences. Hence linalool, and its cyclic derivatives, α -terpineol and linalool oxides, cannot be used to specify a standard enantiomeric ratio. However, linalool is one of the most frequently used compounds in flowery fragrance compositions and it is an intermediate in several large scale processes for the production of vitamin E (Bauer, 1990). Only grossly unusual enantiomeric ratios of linalool could be used to prompt further investigation of suspect essential oil samples.

Lemon oil can be assessed for adulteration by technological treatments by the ee of linalool, terpin-4-ol and α -terpineol. Perpentylated β -cyclodextrin and pemethylated derivative has been successfully applied in the enantioselective MDGC of linalool from essential oils and fruits (Schubert, 1991, Werkhoff, 1991, Bernreuther, 1991), however the possible reversible

hydration of linalool to geraniol or nerol in acidic media can result in an equilibration of linalool's optical activity to yield racemates (Rapp, 1980)

In the oil of tea tree (*Melaleuca alternifolia*) consistent ee of 65:35 (+:-) for terpinen-4-ol and 76:24 (+:-) for α -terpineol were observed for a range of oils analysed (Leach, 1993).

The ratios of enantiomers of 1,8-cineole, α -terpineol, α -pinene and α -phellandrene in eucalyptus oil present as the most obvious lines of investigation.

There are no published papers on the separation of chiral components of boronia oil. Several limitations to single dimensional chromatography arise in the case of this oil. The maximum temperature the chiral column can tolerate is 250°C. Many of the heavier and more polar components of boronia oil will not elute from the column at this temperature and contamination of the column would occur if the extract was introduced to the column without a prior clean-up.

The terpenes in the profile of boronia, which have chiral centres include α -pinene, camphene, sabinene, β -pinene, δ -3-carene, α -ionone, and methyl jasmonate. B-Ionone, which is often commercially synthesised and added to oils to enhance quality, unfortunately does not have a chiral centre so identification of adulterated boronia samples cannot be assessed using enantiomer ratios. A-Ionone, despite having a chiral centre, occurs in boronia at too low a quantity to be an effective indicator of oil adulteration.

In the oil of dill, (+)(4S)- α -phellandrene, (+)(4S)- β -phellandrene, dill ether and carvone occur enantiomerically pure in all investigated plant parts, during all stages of maturity (Faber et al., 1997). The enantiomeric composition of limonene is different for the various plant parts and changes during the development of the umbels. The main components of the herb dill oil are α -phellandrene and dill ether (3,9-epoxy-*p*-menth-1-ene). However there are only traces of these chemicals in the essential oils of dill seeds. The seed oil has a typical caraway odour because of its high (+)(4S)-carvone content. 2-7% of (-)(4R)-carvone has been detected in dill oil (Konig et al., 1990). Faber et al. (1997) achieved stereodifferentiation of carvone on a Duranglass column (30m x 0.23 mm i.d.), coated with

a 0.23 μ m film of 33% heptakis (2,3-di-*O*-acetyl-6-*O*-tert-butyltrimethylsilyl)- β -cyclodextrin.

Some significant differences were detected in the enantiomeric ratios of each of α and β -phellandrene,(+)-sabinene, myrcene and α -pinene in the volatile composition of seeds of *Angelica archangelica* L. from different localities in western, eastern and northern Finnish Lapland (Holm, 1997). Similarly, the relationship of enantiomeric ratios and geographical origin of essential oils has been investigated by Moolenbeck (1997), Kreis (1994) and Faber (1997).

Biogenetic pathways may be elucidated by correlating the enantiomeric ratios of structurally related essential oil components. For example Wust et al. (1997) proposed a pathway for the biogenesis of rose oxide ketone in *Pelargonium* species by correlating the enantiomeric ratios of cis- and trans- rose oxide.

Gas Chromatography of Chiral Chemicals

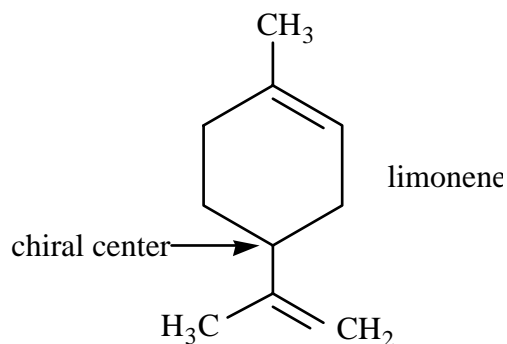
The behaviour of each standard on a chiral column, under a range of conditions was first tested. The standards used are listed below in Table 1.

Table 1. Standard Chiral Chemicals.

Chemical	Source
racemic α -pinene	isolated from essential oils
racemic β -pinene	isolated from essential oils
α -ionone	isolated from essential oils
(+)-limonene	isolated from essential oils
(-)-limonene	isolated from essential oils
(-)-menthol	isolated from essential oils
terpin-4-ol	isolated from essential oils
α -terpineol	isolated from essential oils
jasmonic acid	Sigma-Aldrich
(-)-menthol	Sigma-Aldrich
(+)-menthol	Sigma-Aldrich
(-)-menthone	Sigma-Aldrich
(+)-carvone	Sigma-Aldrich
(-)-carvone	Sigma-Aldrich
(+)-fenchone	Sigma-Aldrich
(-)-fenchone	Sigma-Aldrich
(+)-menthyl acetate	Sigma-Aldrich
(-)-linalool	Sigma-Aldrich

Approximately 3 mg of each standard was dissolved in 10 mL of chloroform. A HP 5890 gas chromatogram equipped with a Hewlett Packard HP Chiral (20% permethylated β -cyclodextrin) column, length 30 m, film thickness 0.25 μm , column ID 0.25 mm, phase ratio 250. The carrier gas was nitrogen. Several oven temperature gradients were trialed.

Limonene (bp₇₆₃ 175.5-176.5°C)



Limonene is a component of dill, peppermint, parsley, fennel, spearmint and boronia oil. It has a pleasant lemon like odour. Both the (+) and (-) form were available through the oil library maintained at the University of Tasmania. Though not enantiomerically pure, the ee was sufficient to be able to distinguish the two forms when separated by GC on the chiral column.

The separation of (-) limonene (84% pure) and (+) limonene (66% pure) was first trialed under the following conditions.

Analytical Conditions

Equipment:	HP5890 GC coupled to flame photometric detector (FID)
Injection:	1 μL , split automatic injections
Column:	30 m HP Chiral (20% permethylated β -cyclodextrin) 0.25mm id, 0.25 μm film thickness, phase ratio 250
Head pressure:	12 psi.
Temp Program:	60°C (held for 10 min), ramped to 80°C at 1°C/min and then at 2.5°C/min to 120°C
Injection Temp:	250°C
Detector:	280°C

Figures 1 and 2 show the retention times were 31.287 and 31.950 minutes for the (-) and (+) enantiomer respectively.

Figure 1. FID Chromatogram of (-) Limonene

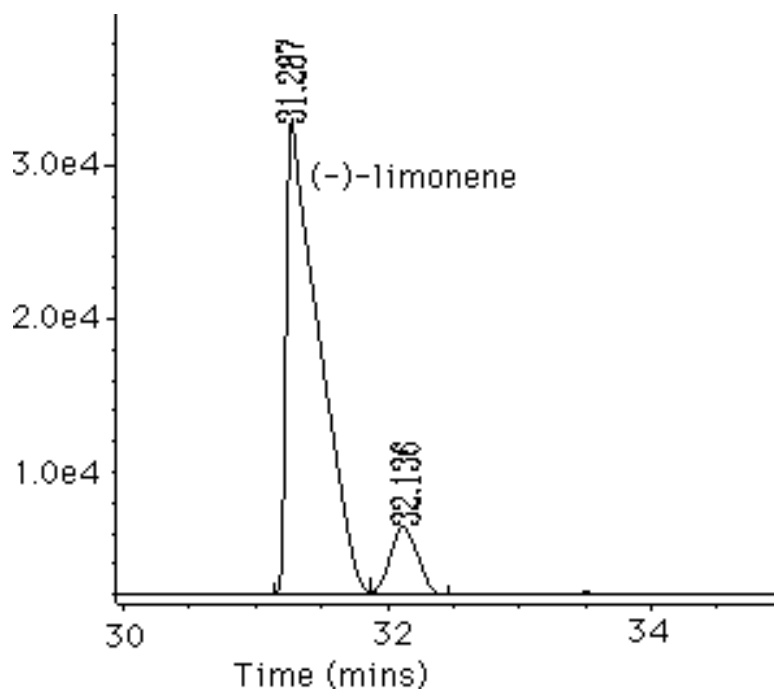
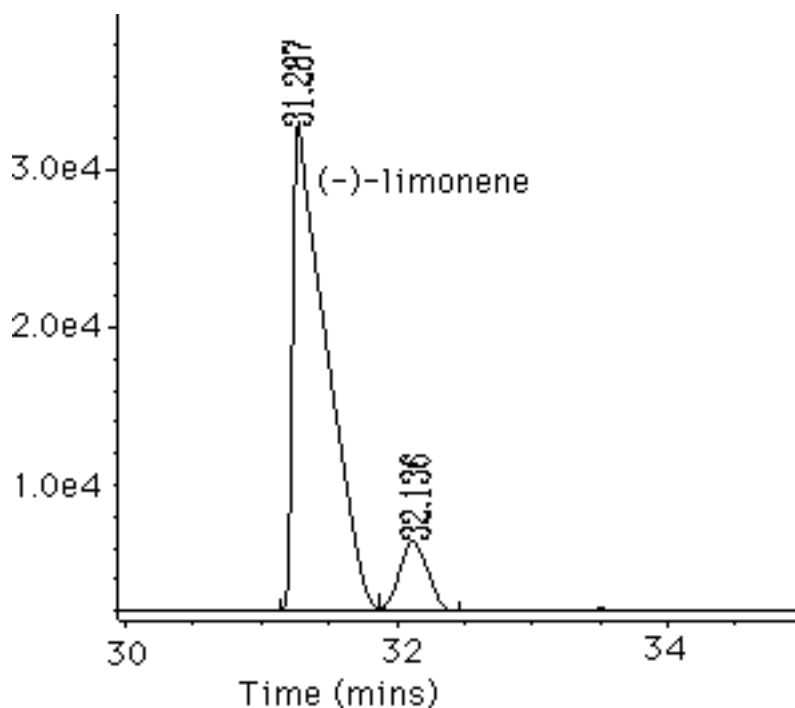


Figure 2. FID Chromatogram of (+)-Limonene



Resolution (R_s) is defined as the separation of 2 peaks in terms of their average peak width at half height (Mosandl, 1992).

$$R_s = 1.77 \left[\frac{At_R}{W_{h1} + W_{h2}} \right]$$

Where

At_R = absolute difference in retention time of the two peaks

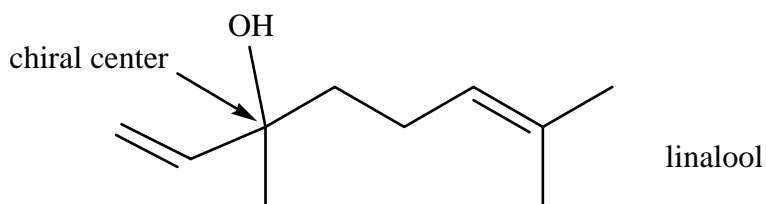
W_{h1} = width of peak 1 at half height

W_{h2} = width of peak 2 at half height

100% separation occurs if the chiral resolution $R_s = 2.50$, in practice $R_s \geq 1.50$ (99.73% separation) is defined as baseline resolution.

When run simultaneously, the separation of the 2 enantiomers using the conditions listed above, achieved an $R_s = 1.42$, just below that defined as base line separation. However, the resolution is sufficient to determine enantiomeric purity when analysed alongside a standard by GC MS or GC FID.

Linalool (3,7-dimethyl-1,6-octadien-3-ol). (bp₇₂₃ 194-197°C)



Linalool is a constituent of boronia, lavender, peppermint oil and many other major essential oils. It is one of the most frequently used compounds to produce a flowery fragrance, as the odour is similar to bergamot or French lavender oil and linalool is often used to replace these oils. As linalool is an important intermediate in vitamin E synthesis, several large scale processes for its production have been developed. (Bauer, 1990).

Initial experiments were conducted using an oil isolated at the University of Tasmania containing both enantiomeric forms of linalool. All equipment and conditions listed in the previous experiment were used in this experiment with the exception of the GC oven temperature gradient. Figure 3 shows the separation of \pm linalool.

Analytical Conditions

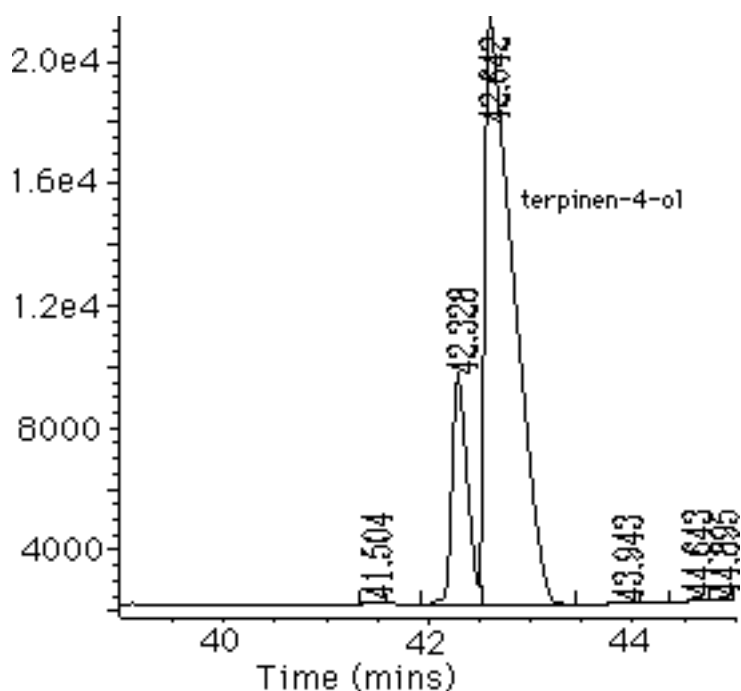
Temp Program: 60°C (held for 10 min), ramped to 120°C at 1°C/min and then at
 2.5°C/min to 160°C held for 10 mins

Terpinen-4-ol is found in many essential oils, including lavender oil. A racemic oil sample had been previously isolated at the University of Tasmania previously and was used in this experiment.

Analytical Conditions

Temp Program: 60°C (held for 10 min), ramped to 120°C at 1°C/min and then at 2.5°C/min to 160°C held for 10 mins

Figure 4. GC Separation of Terpinen-4-ol



Assuming both enantiomeric forms of terpinen-4-ol are present, separation has been achieved with an ee of one form evident. Peak identity must be confirmed using mass spectra, however, which form of the enantiomer is represented by which peak cannot be determined.

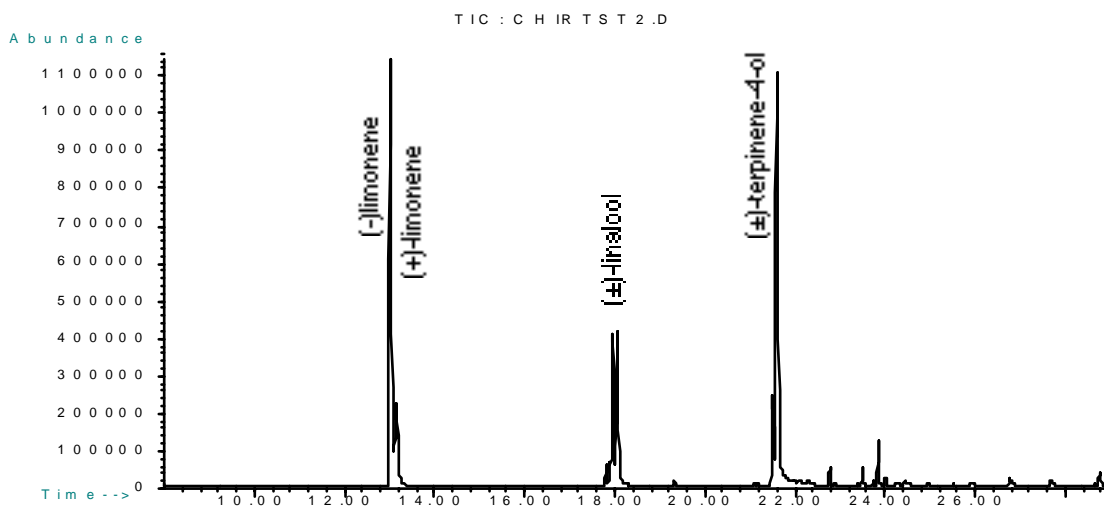
To confirm peak identity, a new solution, containing the limonene, linalool and terpinen-4-ol racemic mixes, was prepared. The solution was injected on a HP 5890 GC, coupled via an open split interface to a HP 5970B mass selective detector (MSD). Several temperature programs were trialed. A fast gradient with an initial temperature of 50°C, then a ramp of 10°C/min to 80°C followed by a 4°C/min ramp to 150°C gave enantiomeric separation of

limonene, linalool and terpinene-4-ol. However, the resolution was affected by this procedure compared to the slower ramps trialed on the GC FID. Figure 5 shows the chromatogram obtained on the column when the initial temperature was 60°C held for 2 minutes with a ramp of 3°C/min to 150°C.

Analytical Conditions

Temp Program: 60°C (held for 10 min), ramped to 120°C at 1°C/min and then at 2.5°C/min to 160°C held for 10 mins

Figure 5. Limonene, Linalool and Terpinene-4-ol Analysed by GC MSD



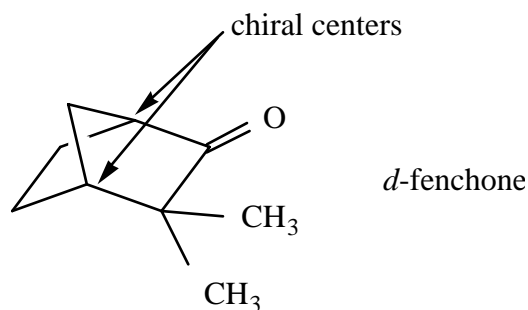
The R_s of (±)-limonene = 1.9, R_s of (±) linalool = 1.7 and R_s of terpinene-4-ol = 2.0. Baseline separation is achieved for all 3 chemicals. Lavender oil contains limonene, linalool and terpinen-4-ol. This oil was analysed under the same conditions for inter comparison. Figure 6 show the chromatogram of lavender oil when run under the GC conditions listed.

Analytical Conditions

Temp Program: 60°C (held for 10 min), ramped to 120°C at 1°C/min and then at 2.5°C/min to 160°C held for 10 mins

Figure 6. Lavender Oil Analysed by GC MSD on Chiral Column

Fenchone 1,3,3-trimethylbicyclo[2.2.1]heptan-2-one (bp₇₆₀ 193.5°C)



Fenchone occurs in fennel oil and in the essential oil of *Lavandula stoechas* L., *Labiatae*. It has a camphor-like odour and is used in foods and perfumes.

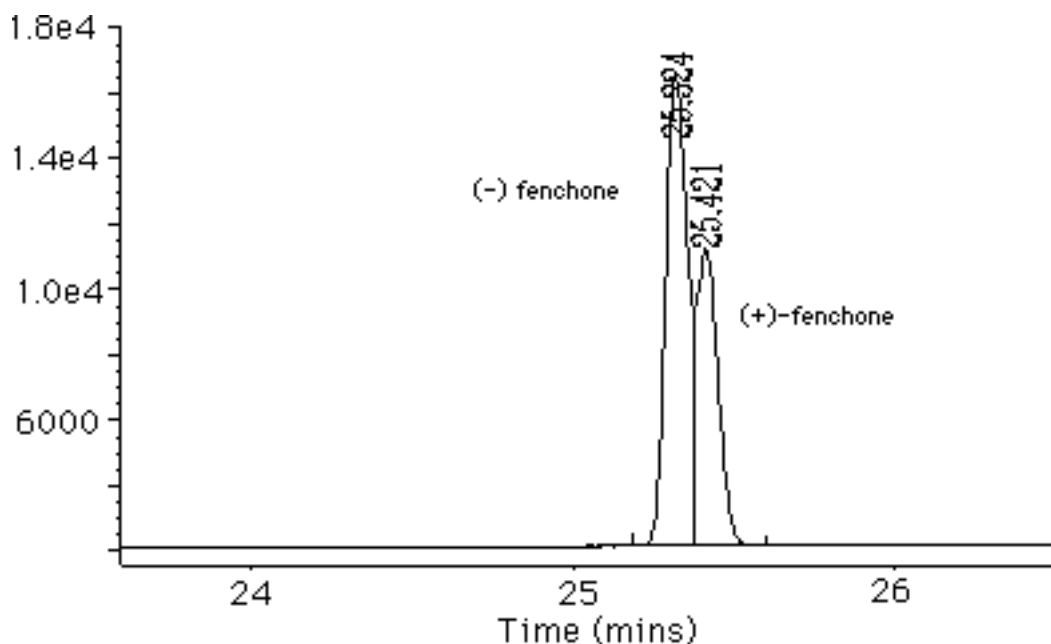
Commercial standards were purchased and each enantiomeric form was run using the temperature gradient previously established.

Analytical Conditions

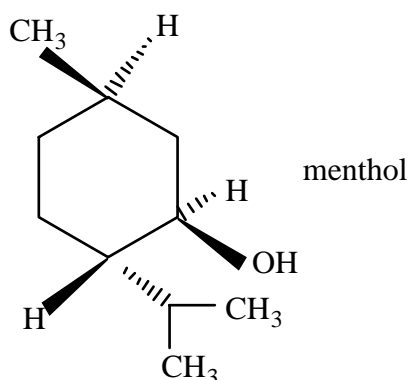
Temp Program: 60°C (held for 10 min), ramped to 120°C at 1°C/min and then at 2.5°C/min to 160°C held for 10 mins

(+) and (-) forms of fenchone eluted at 25.523 and 25.356 minutes respectively when run separately. A mixture of the 2 isomers showed little separation. Several slower temperature gradients were trialed, with little success. Figure 7 shows the chromatogram of (\pm) fenchone under the standard ramp listed above.

Figure 7. GC Separation of (\pm)-Fenchone Enantiomers



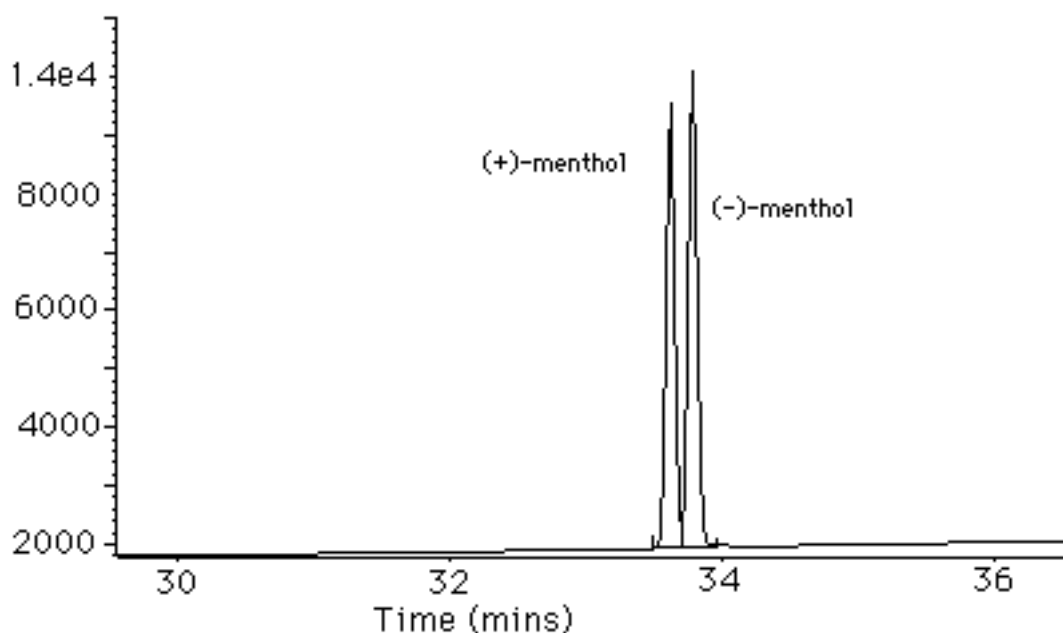
Menthol (1 α ,2 β ,5 α)-5-methyl-2-(1-methylethyl)-cyclohexanol (bp 212°C)



Menthol is obtained from peppermint oil and other essential oils. The crystals of menthol contribute the peppermint taste and odour in liqueurs, confectionary, perfumery, cigarettes, cough drops and toothpaste.

(-)-Menthol had been isolated from peppermint oil by the Horticultural Research group at the University of Tasmania. The (+) isomer was purchased through Sigma Chemicals. The naturally occurring (-) menthol eluted at a temperature of ~143°C. The two enantiomeric were analysed separately. Using the following temperature program conditions listed below, (-) and (+) menthol eluted at 33.688 and 33.802 minutes respectively. The combined (±)-menthol solutions were separated on the HP Chiral (20% permethylated β-cyclodextrin) column with a resolution of $R_s = 1.47$. The results are shown in Figure 8.

Figure 8. GC Separation of Enantiomers of Menthol



Analytical Conditions

Temp Program: 60°C (held for 10 min), ramped to 120°C at 1°C/min and then at 2.5°C/min to 148°C.

Enantiomerically pure (-)-menthol occurs in natural peppermint oil. The synthetic (+)-menthol was used to spike the natural oil to determine if the enantiomers could be distinguished within the matrix of essential oils. Figure 9 shows the gas chromatogram of the natural oil, whilst Figure 10 shows that of the spiked oil. (+)-Menthol adulteration is easily distinguished using this analytical method.

Figure 9. Peppermint Oil Analysed on a Chiral Column

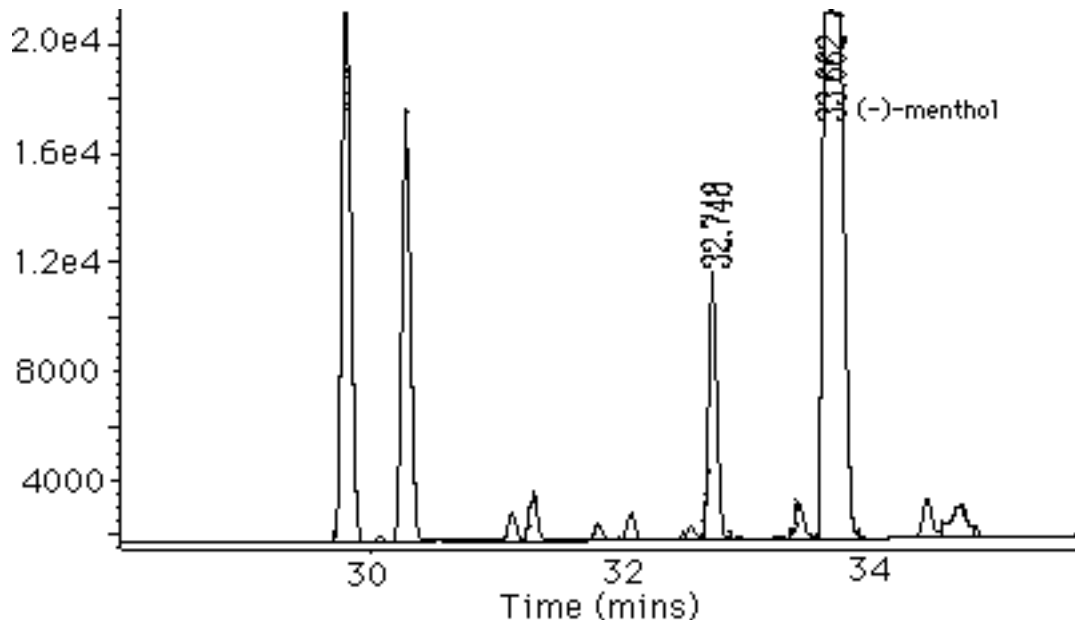
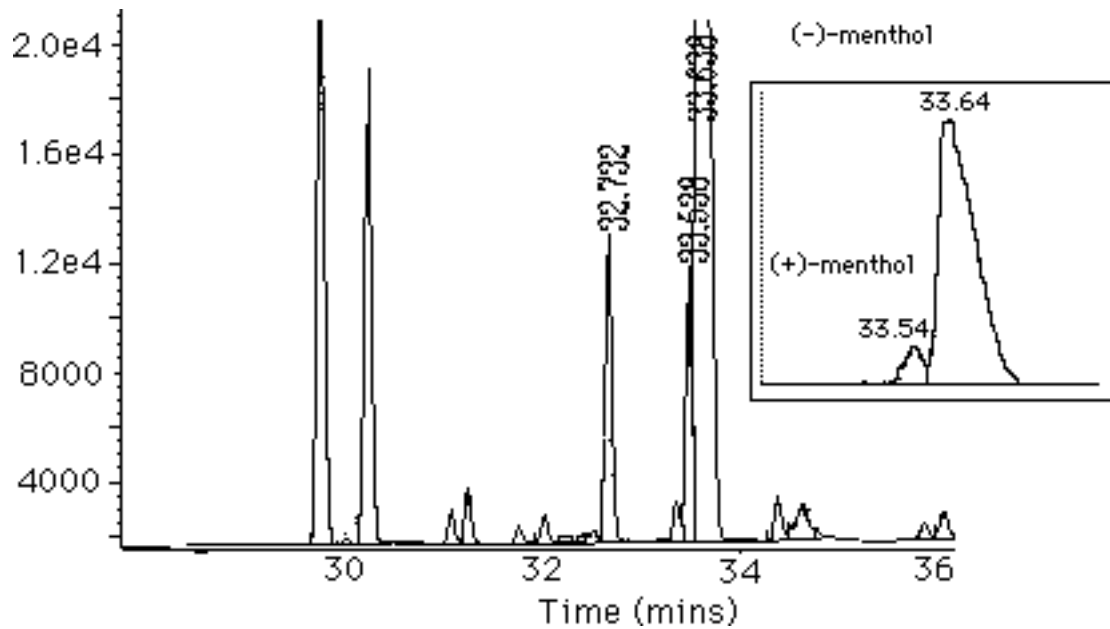
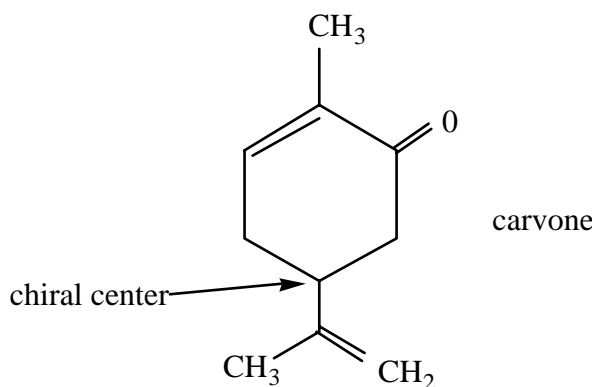


Figure 10. Peppermint Oil Spiked with (+)-Menthol



Carvone 5-isopropenyl-2-methyl-2-cyclohexenone (bp 228-230°C)

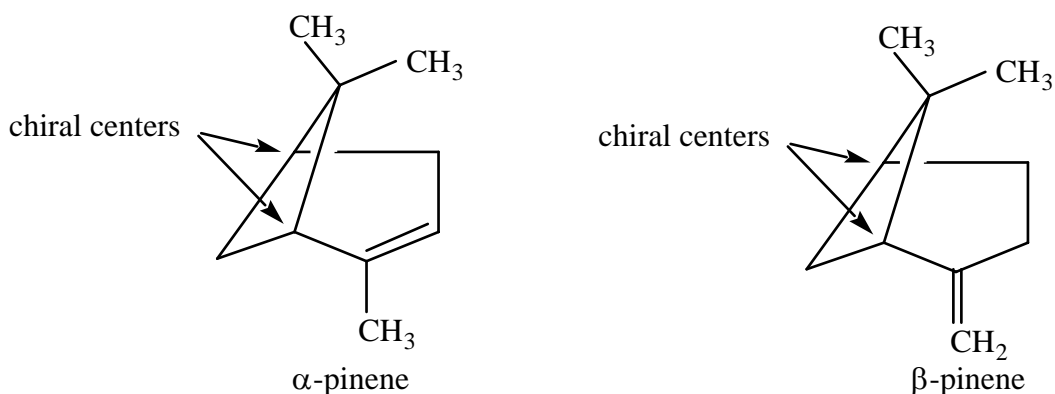


(+)-(4S)-Carvone occurs enantiomerically pure in all investigated plant parts, during all stages of maturity, and in the oil of dill.

Hexane solutions of each enantiomeric form of carvone was prepared and injected onto a HP 5890 gas chromatogram equipped with a Hewlett Packard HP Chiral (20% permethylated β -cyclodextrin) column. With a initial temperature of 60°C, held for 2 minutes, a ramp of 3°C to 120°C, followed by a ramp of 2°C to 160°C, (-) carvone and (+)-carvone eluted at 35.541 and 35.556 minutes respectively. Not surprisingly then, the racemic mix of (\pm)-carvone was not resolved under the same conditions.

Literature reviews reported that the solvent in which combinations of enantiomers are injected can affect the separation. A different dilution phase can change the retention of components because the polarity of the stationary phase varies with the solvent used. Solutions of (\pm)-carvone in methanol, chloroform, ethyl acetate were injected onto the column under a range of conditions. However, no separation of the enantiomers was achieved.

A and β -pinene 2,6,6-trimethylbicyclo[3.1.1]hept-2-ene (bp₇₆₀ 155-156°C)



α -pinene has a characteristic odour of turpentine. Both the (+) and (-) forms are usually present in natural oils. As such, it will not give any insight to possible adulteration using synthetic oils. However, the chirality of the terpenes is relevant to the biological interactions, as the stereochemistry directly affects their bioactivity (Ochocka, 1991). In addition, the ee of the enantiomers of chiral terpenes can be related to geographical origin of the plant material used in oil production.

Obtaining the purified enantiomers of the pinenes is difficult. However, racemic pinene is readily available. Mass spectra and retention indices are sufficient to confirm the presence of pinene but we cannot specify which peaks represent the enantiomeric isomers. We can, however, determine the ee of stereo-isomers without identifying which of the isomers is in excess.

The separation of (\pm) α -pinene was achieved using an initial GC temperature of 60°C, held for 2 minutes with a ramp of 3°C/min to 200°C, held for 10 mins. Figures 11 and 12 show the chromatograms of α - and β -pinene respectively. α -pinene separates into the two (\pm) forms with baseline separation. However, the chromatogram for β -pinene records only one peak. Either there is an ee for one of the enantiomeric forms or (+) and (-)- β -pinene has not been separated. GC MS will provide some insight into the composition of the peak recorded at 17.822 minutes in Figure 12.

Figure 11. Separation of A-pinene Enantiomers

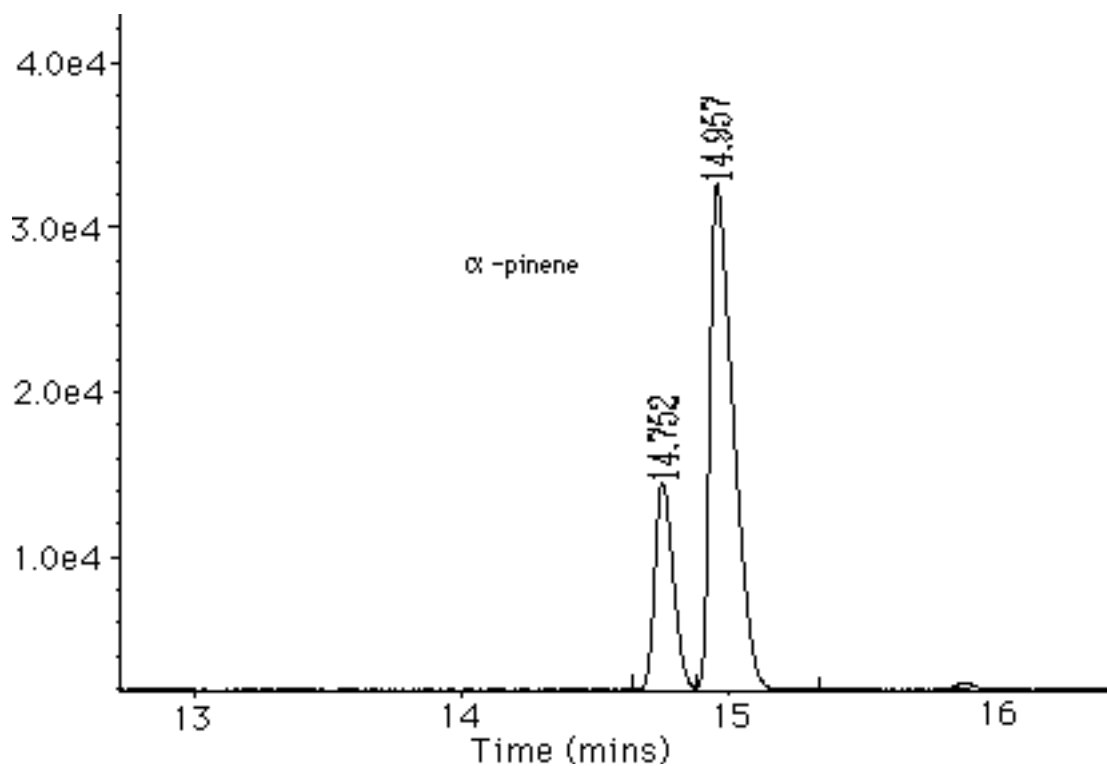
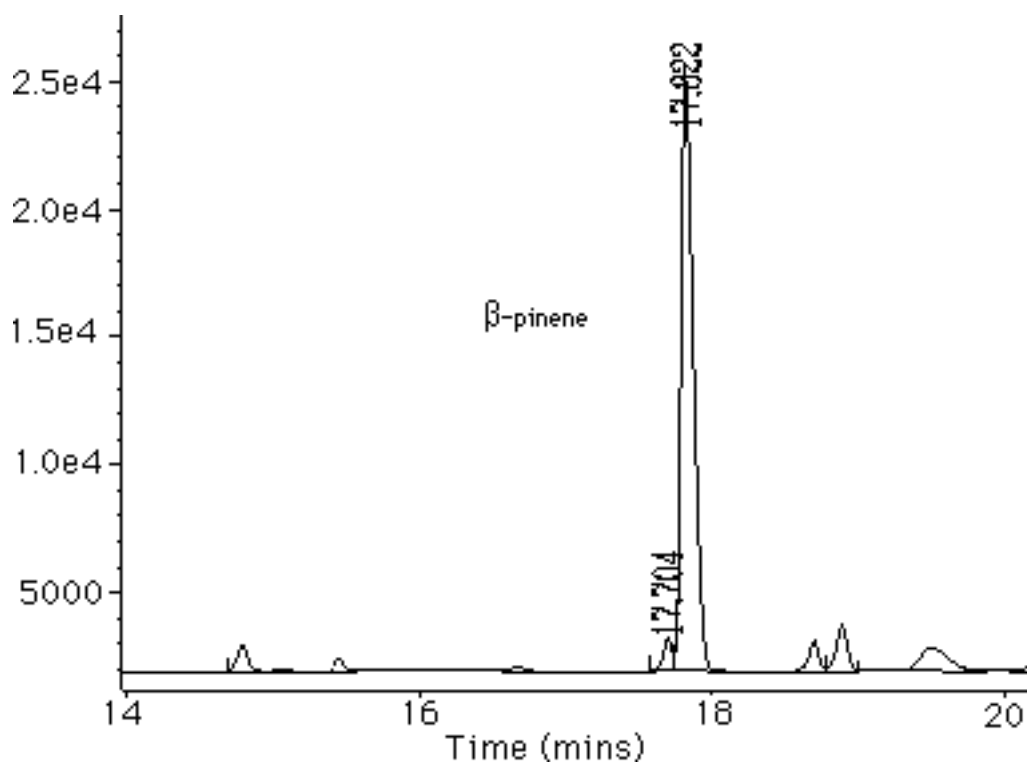


Figure 12. Separation of B-pinene Enantiomers



Chiral Components in Boronia Concretes.

The HP Chiral (20% permethylated β -cyclodextrin) column has an upper maximum temperature of 250°C. The extract of the boronia flower is extremely complex, containing waxes and heavy components with high molecular weights and boiling points. A normal solvent injection of the boronia concrete would introduce waxes and oxygenated terpenes which would remain on the column unless the upper limit was exceeded.

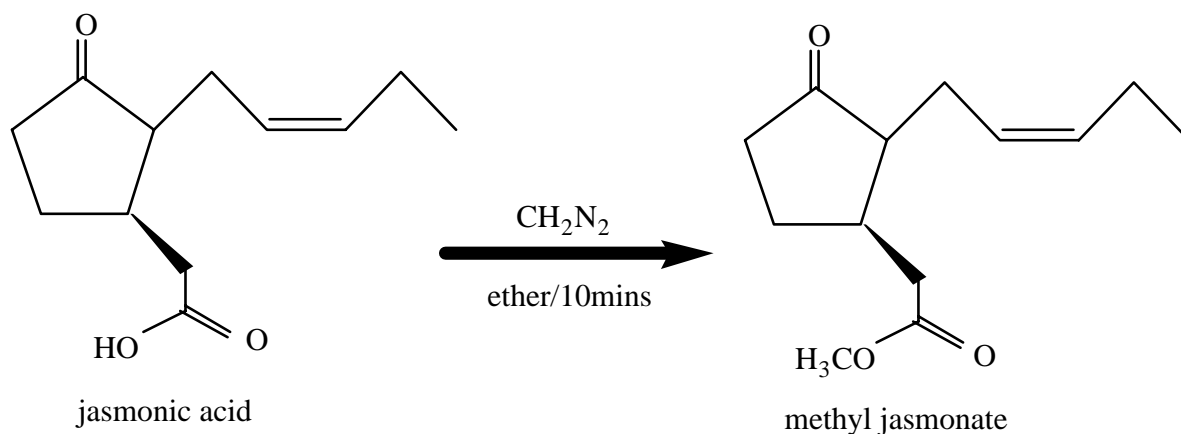
Solid Phase MicroExtraction (SPME) is a relatively new injection technique. A 100 μ M polydimethylsiloxane fibre, encased within a needle, adapted for manual GC injection, is able to rapidly absorb and concentrate the volatiles in the headspace of oils. The fibre will only absorb those chemicals volatile at room temperature and cannot absorb the heavy components of boronia concrete, which threaten to contaminate the chiral column. Once the needle is injected into the GC chamber, heated to 250°C, the volatiles are rapidly desorbed and separated by the normal GC temperature gradient through the column.

As SPME injections will be required to analyse the chiral components of boronia oil, the experimental workup to separate racemic solutions of the standards also used this injection method.

Enantiomers of linalool, limonene, α -pinene and β -pinene were separated using SPME injection with GC separation on the chiral column. The same separations as achieved by automatic injections were achieved. However, the manual SPME injection introduced error in the repeatability of retention times achieved. This parameter alone could not be used to identify the components of the oils and GC MS is required to effectively identify the eluting peaks within essential oils.

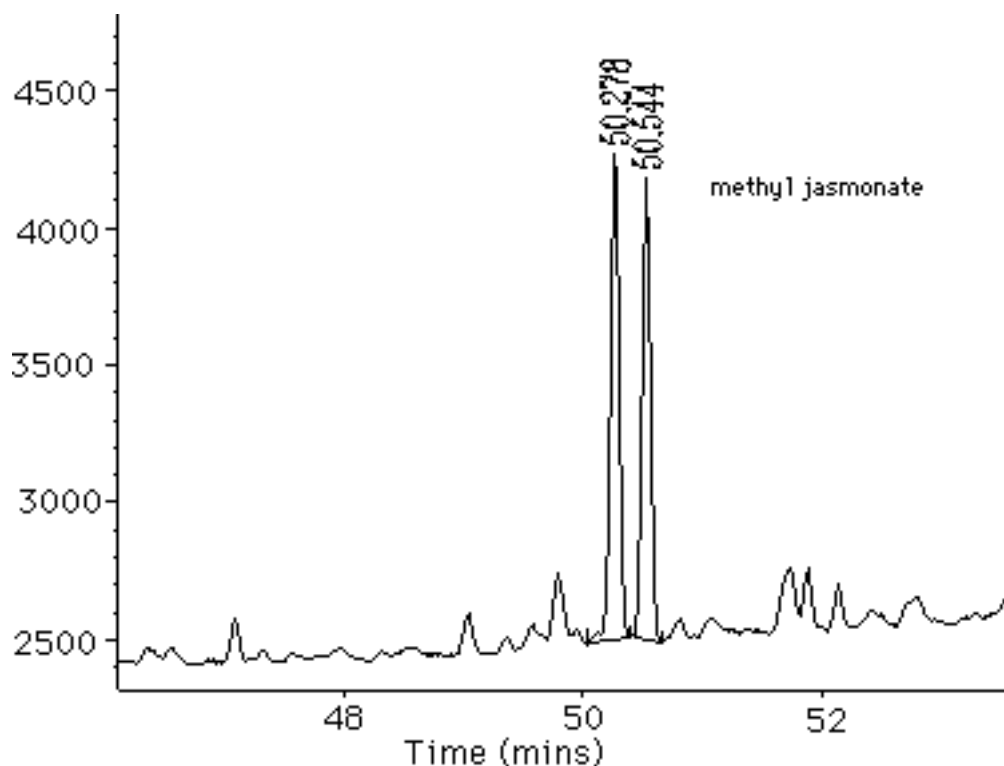
Methyl Jasmonate

Methyl jasmonate was not readily commercially available. Jasmonic acid was purchased from Sigma-Aldrich and derivatised with diazomethane in ether as shown below. The starting material, jasmonic acid, was a racemic mix so analyses will be able to determine whether the enantiomers have been separated, but not the identification of which peak represents which enantiomer.



In the first GC analyses, an automatic injection of the ether solution was performed. The oven was held at 60°C for 3 minutes and then ramped at 3°C/min to 240°C. Two peaks were recorded at 50 minutes. An SPME needle was held over the solution of methyl jasmonate for 2 minutes. The needle was then inserted into the injector chamber of the GC and the internal fibre extruded. The GC run was started manually as soon as the needle entered the injection chamber. The temperature gradient was the same as that used for the automatic injection. The chromatogram in Figure 13 show the 2 peaks recorded at 50.278 and 50.544 minutes. GC MS will be required to confirm the two peaks are the enantiomeric isomers of methyl jasmonate.

Figure 13. Methyl Jasmonate Enantiomers Separated by GC



The methyl jasmonate was injected by SPME into a HP 5890 GC, coupled via an open split interface to a HP 5970B mass selective detector (MSD) in the selective ion monitoring mode (SIM). Figure 14 shows the SIM trace obtained for the 4 diagnostic ions 83, 151, 156 and 224.

Five peaks were recorded in Figure 14. Their mass spectra were identical indicating that each peak represents an isomer of methyl jasmonate. There are 2 chiral centres in methyl jasmonate which would allow the existence of 4 enantiomeric forms. In addition, there is the potential for cis/trans isomerism about the double bond, and around the ring. In conjunction with the enantiomers, this allows for 16 possible isomeric forms to be present. The peaks observed are most likely 5 representatives of these possible configurations.

To determine which of the methyl jasmonate peaks recorded in the standard are also present in boronia concrete, the SPME needle was held over the headspace of a boronia extract. Again the 4 ions 83, 151, 156 and 224 were extracted from the total ion trace and are shown in Figure 15.

Figure 14. Selected Ion Monitoring MS of Methyl Jasmonate

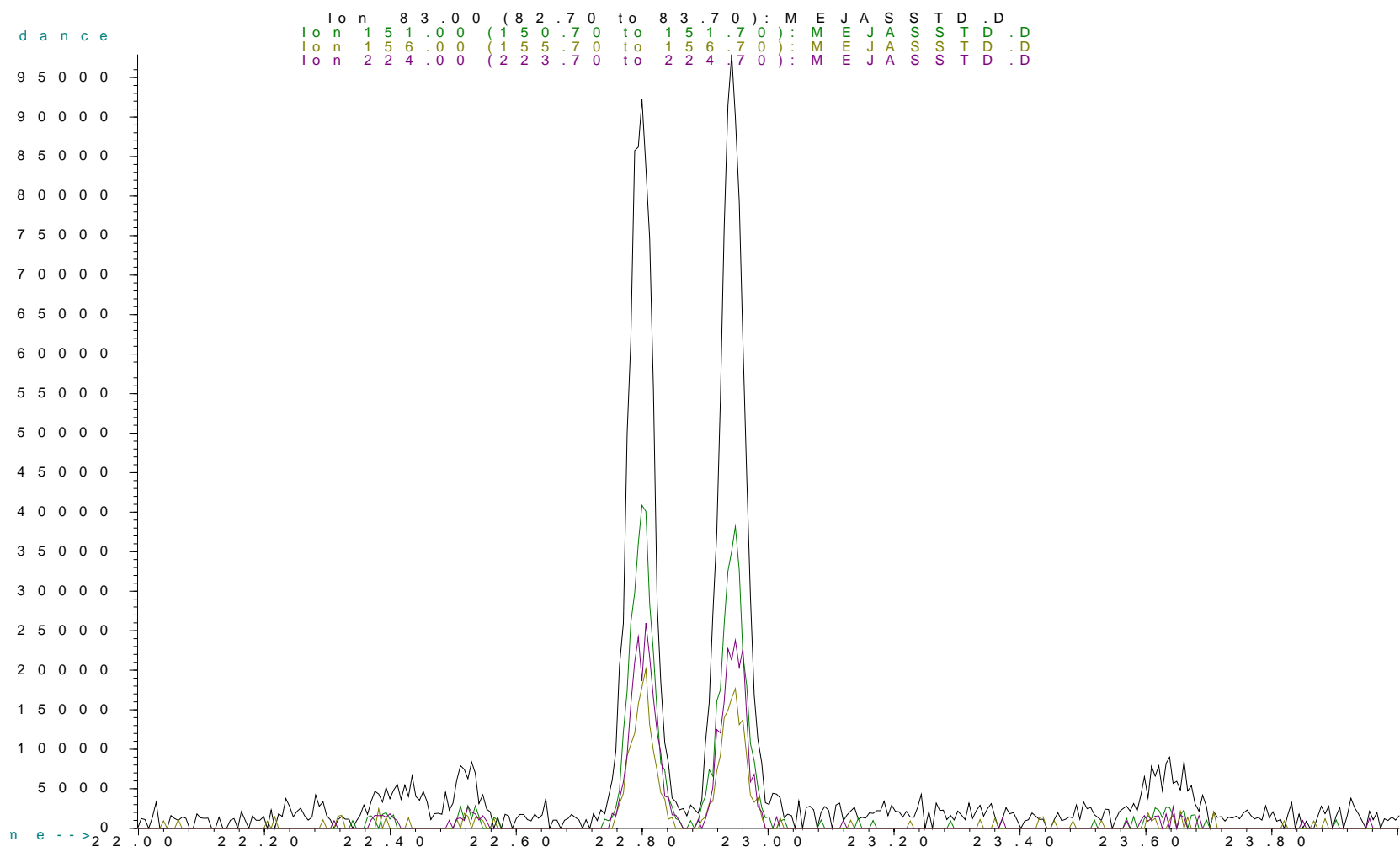
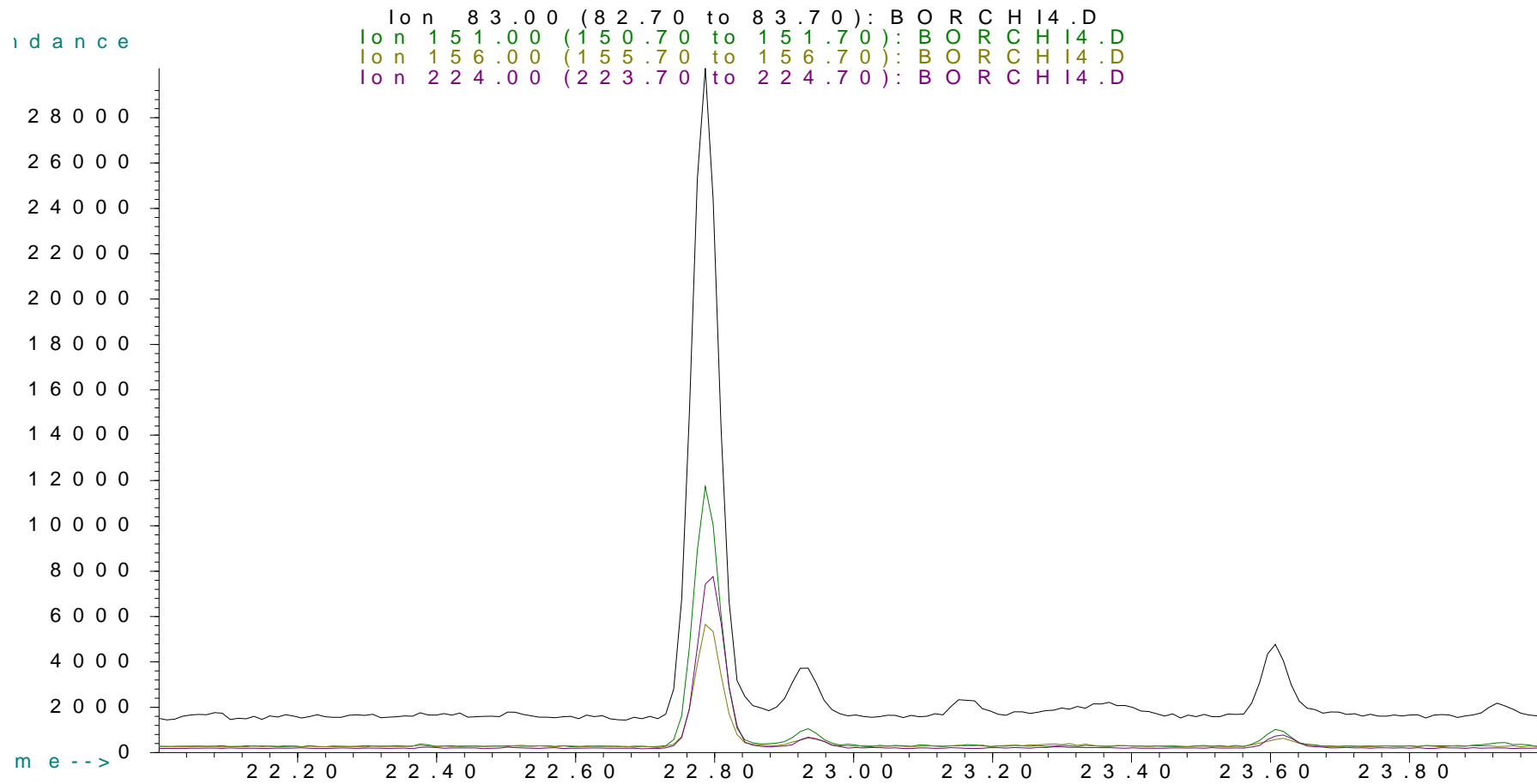


Figure 15. Methyl Jasmonate Endogenous to Boronia Concrete



The major methyl jasmonate peak created by synthesis aligns exactly with that detected in the boronia concrete. In addition it appears there is an ee of one of the enantiomeric forms in the natural product.

Determining Enantiomeric Excesses in Peppermint Oils.

Peppermint oils from China, USA, Japan, Brazil, India, New Zealand and Australia were sub-sampled. 20 mg of each was weighed into GC vials and dissolved in 1mL of hexane. The oils were analysed using the following conditions.

Analytical Conditions

Equipment:	HP5890 GC coupled to flame photometric detector (FID)
Injection:	1 μ L, split automatic injections
Column:	30 m HP Chiral (20% permethylated β -cyclodextrin) 0.25mm id, 0.25 μ m film thickness, phase ratio 250
Head pressure:	12 psi.
Temp Program:	60°C (held for 2 min), ramped to 200°C at 3°C/min
Injection Temp:	250°C
Detector:	280°C

Mint oil of natural origin contains enantiomerically pure (-)-menthone, (+)-isomenthone, (-)-menthol and (-)-menthyl acetate. All investigated *Mentha* species are differentiated with regard to their enantiomeric distribution of (\pm)- α -pinene, (\pm)- β -pinene and (\pm)-limonene [1]. The enantiomeric ratios of α -pinene and β -pinene were compared to determine the feasibility of using single dimensional GC to establish enantiomeric excesses characteristic of the geographical origin of oils. Table 2 lists the results obtained.

Table 2. Enantiomeric Ratios in Peppermint Oil.

Country of Origin	Sample Name	A-pinene ee	B-pinene ee
China	china	bdl	bdl
USA	Suisse Aug 82	49:51	74:26
Japan	Takasago-Japan	44:56	48:52
Japan	Ogawa & Co.-Japan	47:53	42:58
Japan	R.C. TREATT & Co. Yakima Japan	49:51	43:57
Brazil	Brazilian dementholated	71:29	49:51
USA	Bush Boake Allen Franco-Mitcham	bdl	bdl
India	CP-India	52:48	44:56
USA	Bush Boake Allen -Fritzche	41:59	50:50
NZ	Peveler Mint co. Ltd. New Zealand	46:54	49:51
Australia	EOT- Victorian 1978	47:53	50:50
Australia	EOT-Tasmanian 674	48:52	37:63

Determining Enantiomeric Excesses in Boronia Extracts In Tasmania

Several clones have been developed in the Tasmanian boronia industry which produce high yields, are disease resistant, and have high levels of β -ionone. An experiment was conducted to determine if the enantiomeric distribution of some of the chiral components of boronia concrete could be used to distinguish the clones.

Representatives of each of 4 clones were selected and 10-20 mg of the boronia concretes were sub-sampled into GC vials and sealed. Samples were absorbed onto a SPME needle and manually injected using the following conditions.

Analytical Conditions

Equipment: HP 5890 GC, coupled via an open split interface to a HP 5970B mass selective detector (MSD).
Injection: manual SPME
Column: 30 m HP Chiral (20% permethylated β -cyclodextrin) 0.25mm id, 0.25 μ m film thickness, phase ratio 250
Head pressure: 12 psi.
Temp Program: 60°C (held for 2 min), ramped to 200°C at 3°C/min
Injection Temp: 250°C
Detector: 280°C

One of the chemicals to be analysed is methyl jasmonate, which has a low volatility compared to the monoterpenes, α and β -pinene. The needle will absorb the high volatility chemicals at a significantly faster rate than that for methyl jasmonate. Initial results indicated that for sufficient quantities of methyl jasmonate to be absorbed, the needle required an exposure time of several minutes. However, this resulted in large quantities of the monoterpenes being absorbed, such that the peaks recorded for chemicals, such as pinene and limonene, were overloaded. As such, two injections were required. The first was for the analyses of methyl jasmonate. The manual injection was performed, splitless, onto a column held at 30°C for 2 minutes then ramped at 20°C to 150°C followed by a ramp of 5°C to 240°C.

The lower boiling point components were analysed by manual split injection.

Despite the detection of an enantiomeric excess in the boronia concrete analysed in the method development, enantiomeric purity of methyl jasmonate was detected in every clone analysed. As such, the ee is not suitable for distinguishing boronia clones.

The enantiomeric ratios of α -pinene, camphene, β -pinene and β -phellandrene were calculated for the four boronia clones, known as clones 250, 5, 17 and 3. The assignment of the absolute configuration of the chemicals is beyond the scope of this project. The peak which elutes first in the chromatogram of an enantiomeric pair was arbitrarily assigned as peak 1, whilst the peak with the longer retention time was labelled peak 2 for the purpose of comparison in this report. Table 3. shows the ratios calculated for peak 2:1.

Table 3. Ratio of the Area of the Enantiomeric Pairs of A-pinene, Camphene, B-pinene and B-phellandrene in Boronia Clones.

	α -pinene	camphene	β -pinene	β -phellandrene
250	76:24	36:64	5:95	17:83
250	92:8	19:81	30:70	8:92
250	84:16	33:67	0:100	14:86
5	82:18	14:86	0:100	17:83
5	75:25	80:20	100:0	13:87
17	18:82	57:43	85:15	5:95
17	15:85	60:40	85:15	6:94
17	20:80	72:28	0:100	9:91
17	19:81	62:38	78:22	8:92
3	28:72	31:69	69:31	21:79
3	77:23	85:15	100:0	13:87
3	81:19	86:14	100:0	9:91

Discussion

Objective:

To appraise the suitability of chiral gas chromatography columns for the elucidation of the enantiomeric composition of essential oils

A review of the major essential oil products exported from Australia, identified lavender, lemon, boronia, peppermint, dill, tea tree, eucalyptus and caraway oil as being some of the extracts of immediately relevancy to the industry. The chemical composition of these oils was reviewed. Components with a chiral centre were identified for each oil and these are listed in Table 4.

The use of enantiomeric ratios in distinguishing the geographical origin has been reported for mint oils (Mosandl, 1991), lavender oil (Mosandl, 1990, Kreis, 1992) lemon oil, (Schubert, 1991, Werkhoff, 1991, Bernreuther, 1991), amongst others. There are no published papers on the separation of chiral components of boronia oil. Enantioselective columns have been used to elucidate biosynthetic pathways by correlating enantiomeric excesses of structurally related terpenes. Adulteration of natural oils with synthetic derivatives can be detected for oils which produce components with otherwise enantiomerically pure forms. However, several limitations of the chiral assessment of essential oils exist.

A great variety of well-defined authentic samples must be examined to provide a statistical background (Faber et al., 1997).

Chiral stability of many chiral chemicals in essential oils depends on the stage of fruit ripeness, pH value of plant material and technological influences during processing or storage of food stuffs. The enantiomeric ratios of α -terpineol, linalool and linalool oxides change throughout the maturation of plant material. The vigorous treatments plant materials under go in the production of oils, such as steam distillation, solvent extraction and dry down under conditions of vacuum and raised temperatures, may all contribute to the

confusion of the original enantiomer ratios. This is exemplified by the production of (S)-(+)-linalool by excessively long hydro-distillations of lavender species (Kreis, 1992).

Table 4. Chemical Composition of the Essential Oils Reviewed

Essential Oils	Chiral Components
<i>Mentha</i> Species	(-)-menthone (+)-isomenthone (-)-menthol (-)-menthylacetate pulegone
Lavender oil	linalyl oxides linalool linalyl acetate borneol bornyl acetate α -terpineol trans nerolidols
Lemon oil	linalool terpinen-4-ol α -terpineol
boronia extract	α -pinene camphene sabinene β -pinene delta-3-carene α -ionone methyl jasmonate
herb dill oil	α -phellandrene (+)-(4S)-carvone (2-7%) (-)-(4R)-carvone dill ether (3,9-epoxy-p-menth-1-ene)
tea tree oil	α -pinene β -pinene α -phellandrene α -terpineol limonene β -phellandrene terpinen-4-ol
eucalyptus oil	α -pinene α -phellandrene limonene α -terpineol 1,8-cineole

In addition, storage conditions can affect the chemical profile of essential oils. Racemates of natural origin can be generated by non-enzymatic reactions such as auto-oxidation and photo-oxidation. Presentation of products in transparent containers, under less than ideal

conditions of temperature and light, may result in a dramatically altered chemical profiles. Although it may be assumed that producers and retailers alike, would ensure that oils are stored under the best conditions practicable, this factor may make the standardisation of oil composition difficult.

Only chiral volatiles of high optical purity and with characteristic and small ranges of ee values can be used to validated origin of essential oil components or detect the blending of natural and synthetic chiral flavour compounds. This criteria is easily met for chemicals such as (-)-menthol in peppermint and (R)-(-)-linalyl acetate in lavender oil. Both chemicals not only occur enantiomerically pure naturally, but are present as a major component in the oils and confer the aroma and properties characteristic of the respective oils. On the other hand, any worthwhile adulteration of boronia oil would probably include synthetic β -ionone, which has no chiral centre and so cannot be used as a diagnostic marker. Every component of each oil subject to this type of a study needs to be assessed individually in terms enantiomeric excesses for delineation of geographical origin of the oils, and in terms of the importance of the contribution to the quality of the oil with regard to detection of adulteration.

Finally, the recent advances in the development of chiral columns has meant that research to date has been limited. The mass spectra of enantiomers are identical and there is a paucity of data on the relative retention times and Kovat indices of chiral chemicals on chiral columns. Few research programs have the resources to isolate the chemicals within the complex extracts of essential oils, let alone undertake the separation of each of an enantiomeric pair. As such, peak identification in the gas chromatographic analysis of chiral components, within the matrix of essential oils, is often reliant on the availability of commercially produced standards.

Nevertheless, the systematic evaluation of origin specific enantiomers has proved to be a valuable criterion for differentiation natural flavour compound from those of synthetic origin.

The type of column most suitable for application in essential oil analysis was investigated. The use of cyclodextrin derivatives for the stationary phase in enantioselective gas chromatography columns is now well established. Although the exact mechanism underlying the separation of chemicals differing only by the orientation of atoms about one carbon atom, has not been determined, it is suggested that the steric effects influence the enantiomeric differentiation. The increase in enantioselectivity observed with the increasing size of the alkyl side chains in γ -permethylated cyclodextrins, when analysing chemicals with increasing space requirements, highlights the importance of the inclusion effects for enantiomeric separation, allowing a greater interaction between the mobile and stationary phases (Steinborn, 1995).

The enantiomeric differentiation can be so influenced by steric effects on cyclodextrin phases that the effect of the position of even a methyl group can influence the effectiveness of separation.

Many types of chiral columns are now produced commercially. Papers reviewed, used a range of types, often using achiral columns in tandem with the chiral varieties. Multi-dimensional gas chromatography (MDGC) was not a feasible option within the time and budget constraints of this project. B-cyclodextrin chiral columns were selected for use in this study because of their broad applicability to many chemical types. A Hewlett Packard column was selected with a phenyl based polymer interdispersed with β -cyclodextrin.

Objective:**To undertake the separation of racemic mixes of standards using chiral columns.**

Results indicated that 20% β -cyclodextrin columns on phenyl based polymers have wide applicability in essential oils, with baseline separation achieved for a great number of enantiomers investigated. Resolution was improved with slower temperature programs. Not all chemicals investigated were separated on the chiral column. There was no obvious structural feature unique to these chemicals which could explain the lack of resolution. The solvent in which standards were injected did not affect the resolution.

SPME injection is suitable for the introduction of standards into the chiral column with no loss of resolution despite the lack of a cold trap to concentrate the volatiles at the head of the column. However, SPME is a manual injection and the loss of automation resulted in some variability in the retention times such that this parameter could not be used for peak identification. As such SPME injection requires detection using mass spectra.

Objective:**To transfer methodology to the analysis of oil and extract samples.**

Separation of enantiomers of terpenes within the matrix of essential oils was achieved. Mass spectra was used for peak identification. Standards were required to confirm identification as there is little available data on the Kovat indices of chemicals specific to chiral columns. Where enantiomerically pure standards were not available, the absolute configuration of peaks separated could not be specified. Despite this, resolution was sufficient to allow for the comparison of enantiomeric ratios.

Oils from a range of geographical locations and from different clones were analysed to determine the effectiveness of the methods developed to identify enantiomeric excesses. Insufficient data was available to draw any conclusions about the relationship of the ee of the terpenes to geographical origin of peppermint oil. Results do show, however, that the

use of chiral columns using GC FID is suitable for the assessment of this aspect of essential oil research.

The results detailing the chiral composition of boronia clones in Table 3 indicate that the ee of camphene may be suitable to distinguish clone 250 from clone 17. In each of the relevant oils analysed, the second of the enantiomers of camphene to elute on the chiral column of clone 250 consistently was in excess of the first, more quickly eluting racemate of the pair. The reverse of this was true for the clone 17 oils analysed. A larger database, with the analyses of oils produced over several seasons, may show other trends useful for the standardisation of boronia oils.

The use of GC FID has limited application, as does mass spectroscopy in the total ion monitoring mode when using SPME injection. The often complex profiles of essential oils, analysed by gas chromatography, is further complicated using chiral columns by the doubling of peak numbers for optically active components. Peak identity needs to be confirmed by mass spectra, with overlapping peaks delineated by extracting the traces of diagnostic ions from the total ion chromatograms. Alternative columns should be investigated towards achieving resolution of optically active chemicals such as carvone and fenchone.

Over all, single dimension gas chromatography methods proved to be robust and reproducible. The obvious direction for further investigation within this field is the application of multi-dimensional gas chromatography (MDGC). The complexities of essential oils test the limits of a single column system. Separation of the major components is best achieved on a achiral column with heart cutting techniques employed to transfer relevant sections of the essential oil profile to a chiral column.

MDGC can be achieved with a single oven system were the pre-column and the chiral column are linked with a heart-cut valve. The cut is focussed on a cold trap positioned before the analytical column and eluted on a second temperature program. However, this system is limited by the upper limit of the chiral column.

Maximum flexibility is achieved when two gas chromatography ovens are used in tandem, linked by a heated interface. A 'T' valve is used to cut the portion of the chromatogram containing the compound of interest from the first column, transferring the section to the chiral column in a second oven via the interface. The heartcut is focussed on the second column using a cold trap.

The configuration of a MDGC system can be costly. The timing and transfer of the heartcut is critical to obtain reproducible results. The configuration requires a series of valves and timers. A review of the technologies identified MDS 2000, as a cost effective option for the adaptation of existing systems., produced by the company 'SGE', which combines all the pneumatic controls and circuitry into a free-standing module. Along with electronic pressure control, a powerful and adaptable system could be assembled with the gas chromatograms standard to most analytical laboratories.

Implications and Recommendations

The results presented in this project are preliminary and it is too early in the research process to make definitive recommendations. Directions in research, however are clear:

- Test the effectiveness of MDGC systems
- Obtain enantiomerically pure standards by isolation from natural sources or purchase commercial standards where available
- Extend the database detailing retention characteristics of enantiomers on chiral columns

Extend the database recording the enantiomeric distributions of chemicals in essential oils.

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