EXTRACTION, ANALYSIS, AND BIOLOGICAL SCREENING OF *CALLITRIS* SPECIES ESSENTIAL OILS.

by

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Dedication

Dedicated to my beloved parents and almighty God.....

Abstract

The natural products have provided considerable value to the pharmaceutical industry over the past century and its demand is steadily increasing. This is mainly attributed to several factors; unachieved therapeutic needs, demand for bulk supplies and great impact of herbal remedies in the global market. Essential oils or essences have an extraordinary range of pharmacological activities including antiallergic, antiinflammatory, antimutagenic and antimicrobial activities. Essential oils find uses in pharmaceutics, cosmaceutics and aromatherapy. Essential oils can be obtained by cold press extraction, steam distillation, supercritical fluid extraction and solvent extraction. The objectives of the study were to characterise the composition of essential oils from different plant parts of Tasmanian *Callitris* spp *C. rhomboidea* and *C. oblonga*, to identify some of the major unknown constituents in the root oil, to investigate the composition of the solvent extracts, to investigate the biological activities of the *C. rhomboidea* and *C. oblonga* SD oils and extracts against a range of bacteria and fungi, to investigate the release of essential oils from *C. rhomboidea* roots *in situ*, and to investigate the allelopathic activities of *C. rhomboidea* and *C. oblonga* root and leaf oils.

Volatile fractions from the roots, leaves, bark and fruits of *Callitris oblonga* and *Callitris rhomboidea* were obtained by steam distillation (SD) and solvent extraction with petroleum ether (PE) and dichloromethane (DCM). Essential oil composition was analysed by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionisation detection (GC-FID). Thirty six compounds, representing 85% of the steam distillate from *C. rhomboidea* roots, and 45 compounds representing 90% of the steam distillate from the *C. oblonga* roots were identified. Sesquiterpene hydrocarbons constituted the major portion of *Callitris* spp. root oils. The main identified constituents found in the SD root oil of *C. rhomboidea* were longiborneol (23%) and longifolene (5%); the main constituent from SD root oil of *C. oblonga* was columellarin (30%). In the SD fraction of the *C. rhomboidea* leaf α-pinene (42%), geranyl acetate (12%), citronellyl acetate (7%) and neryl acetate (6%) were the major constituents. *C. oblonga* SD leaf oil contained significant amount of α-pinene (45%), isopulegol (6%) and pulegol (2%). The major components in the fruit and bark oil of the *Callitris* spp. were monoterpenes such as α- and β-pinenes.

Solvent extracts contained a high percentage of diterpenes. However the major compounds analysed by GC-MS from the SD fractions of the respective plant organs were also present in their respective solvent extracts.

Experiments were performed on collected roots and leaves ('in vial' experiments) and on intact roots of *Callitris* plants to investigate the release of volatiles utilising solid phase microextraction (SPME). A soil probe designed and manufactured inhouse was used for the study of volatiles evolved from the intact roots. Model experiments using known quantities of volatiles adsorbed onto sand were performed to determine the efficiency of the soil probe. Analysis of 'in vial' SPME sample experiments of roots demonstrated a release pattern of volatiles characterstic of the respective root oils; while leaf sample 'in vial' analysis did not show a characterstic pattern of their respective leaf oils. *In situ* samplings were done from 2 trees growing at different locations. GC-MS analysis of both samples demonstrated the presence of petrochemical-like hydrocarbons. The samples collected from the second site demonstrated the presence of monoterpene hydrocarbons. These monoterpenes were present in the same ratios as in the steam distilled root oils.

Antimicrobial screening of *Callitris* essential oils, leaf oil fractions and extracts of different organs of *Callitris* spp. was performed against the bacteria *Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus epidermis*, *Dermatophilus congolensis*, *Proteus mirabilis*, *Escherichia coli* and *Pseudomonas aeruginosa*, and the fungi *Epidermophyton floccosum*, *Microsporum canis*, *Microsporum gypseum*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Trichophyton soudanese*, *Candida albicans* and *Phytopthora infestans*. Oils were screend by a disk diffusion assay technique and the most potent essential oils against a particular organism were tested by a broth microdilution assay. None of the *Callitris* spp. essential oils exhibited potent activity against bacterial organisms. *C. rhomboidea* leaf oil exhibited comparatively high activity against *M. canis*. The oil was fractionated and screened by a broth microdilution assay. The constituents mainly responsible for the antifungal activity were geraniol and citronellol, with MIC values of 0.13-0.06% v/v and 2.0-1.0% v/v respectively.

Antioxidant assays were performed on essential oils and methanolic extracts by the 2,2-diphenyl picrylhydrazyl (DPPH) radical scavenging assay. The oils and extracts were compared against gallic acid standards. The tested *Callitris* essential oils showed very weak antioxidant activity.

Lettuce seeds were used to study the allelopathic effects of essential oils. *Callitris* oils at a concentration of 20, 4, and 1 micro litre per 20 ml, exhibited strong allelopathic effects on the growth of lettuce plants.

The major unknown constituent of the *C. oblonga* root oil was identified as Columellarin. The composition of the solvent extracts were analysed and established. The SPME experiments demonstrated that the *C. rhomboidea* roots released volatiles *insitu*. Root oil novel to the Cupressaceae family displayed little or no activity against the tested fungal and bacterial organisms. *C. rhomboidea* leaf oil exhibited useful activity against *Microsporum canis*; the antifungal activity was primarily due to the presence of geraniol and citronellol. Lettuce seed bioassay demonstrated that *Callitris* oils possess allelpathic activity against the gowth of plants.

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Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any other tertiary institution.

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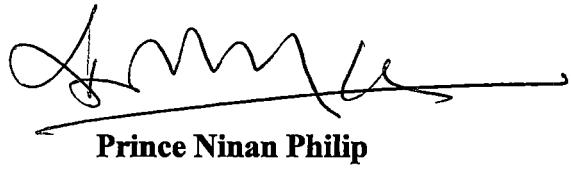

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ACRONYMS AND ABBREVIATIONS

°C	degree Celsius
ACO	Australian Cypress Oil Pty Ltd
AIDS	acquired immunodeficiency syndrome
AMH	Australian Medicine Handbook
amu	atomic mass unit
AR	analytical reagent
ATCC	American Type Culture Collection
BHI	brain heart infusion
BSC	basal sarcoma cells
C	carbon
cfu	colony forming unit
cm	centimetre
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CVS	cardiovascular
DCM	dichloromethane
DMAPP	dimethylallyl pyrophosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl
DVB	divinyl benzene
FDA	Food and Drug Administration
FID	flame ionisation detector
Fig	figure
FPP	farnesyl pyrophosphate
g	gram
GA	gallic acid
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GFPP	geranylfarnesyl pyrophosphate
GGPP	geranylgeranyl pyrophosphate
GPP	geranyl pyrophosphate
HIV	human immunodeficiency virus
HLB	hydrophilic-lipophilic balance

HPLC	high-performance liquid chromatography
HSV	herpes simplex virus
INRA	L'Institute National de la Recherche Agronomique
IPP	isopentyl pyrophosphate
IR	infrared
KI	Kovats index
l	litre
LC	liquid chromatography
LLE	liquid-liquid extraction
LR	laboratory reagent
m/z	mass to charge ratio
MDMA	methylenedioxymethamphetamine
MeOH	methanol
MFC	minimum fungicidal concentration
mg	milligram
MHA	mueller-Hinton agar
MIC	minimum inhibitory concentration
ml	millilitre
MOPS	3-(N-morpholino) propanesulfonic acid
MPharmSc	Master of Pharmaceutical Science
MS	mass spectrometry
MS	mass spectrum
MW	molecular weight
NCCLS	National Committee for Clinical Laboratory Standards
ND	not determined
NIST	National Institute of Standards and Technology
nm	nanometer
NMR	nuclear magnetic spectroscopy
PDMS	polydimethylsiloxane
PE	petroleum ether
ppm	parts per million
RPMI	Roswell Park Memorial Institute medium
RT	retention time

RF	retention factor
SD	steam distillation
SD	standard deviation
SDA	sabouraud dextrose agar
SFE	supercritical fluid extraction
SPE	solid phase extraction
SPME	solid phase microextraction
Spp	species
TIC	total ion current
TLC	thin layer chromatography
UC	unknown compound
UV	ultraviolet chromatography
UV	ultraviolet
VF	varian factor
VOC	volatile organic compounds
ZI	zone of inhibition

CONTENTS

CHAPTER 1

General introduction of *Callitris* spp. plants

1.1 NATURAL PRODUCTS AS THERAPEUTIC REMEDIES

The use of natural products, especially plants, for healing, dates back to the ancient civilizations. Natural products have played a prominent role in traditional medicine systems like Chinese, Ayurveda and Egyptian, which are effective even today. Natural products can be obtained from plants, microorganisms, marine organisms, vertebrates and invertebrates (Cragg *et al.*, 1997). Natural product research for identification of a lead molecule for drug development increased worldwide through the 1990s with the re-emergence of infective diseases for which no effective therapies are available and the development of resistance by many pathogens to currently used drugs (Cragg *et al.*, 1997).

Many top selling drugs of last century have been prepared from natural products or have been remodelled from natural products, such as vincristine from *Vinca rosea*, pethidine and morphine from *Papaver somniferum* (Cragg *et al.*, 1997; Sarkar *et al.*, 2006). To put it more precisely Cragg and colleagues showed that of the 520 new drugs in various classes that were approved by the United States Food and Drug Administration (FDA) from 1983-1994, 30 entities were of natural products and 173 were either semi-synthetic or remodelled from a natural pharmacophore (Cragg *et al.*, 1997).

The period from 1940 to 1970 is often considered as the "Golden age of Antibiotics" due to the serendipitous discovery of penicillin by Alexander Fleming in 1928. Penicillin became the lead molecule for the synthesis of various other penicillins and other analogues. Since that day natural products has taken its importance in various fields of medicine (Newman *et al.*, 2000).

Although natural products provided the majority of lead molecules, the decline in the output of research and development programmes due to the mounting cost of drug development and the overcaution exercised by the FDA in drug approval has caused a reduction in the development of new leads by pharmaceutical companies by 'trial and error' methods (Newman *et al.*, 2003).

Some challenges in drug development from natural products include preserving the biological diversity of the rainforests, developing medicinal plant germ banks, developing global information on plants, assuring the safety and efficacy of traditional medicines, harmonization within major countries of the world with respect to the regulations regarding access to the biome (intellectual property rights), optimizing the chemical diversity of natural products, enhancing drug discovery technology and developing personnel and infrastructure for conducting the investigations (Cordell, 2003). Difficulty in standardisation of natural products due to the lack of separation methodologies, has always led to quality related problems (lack of consistency, safety and efficacy), overshadowing the genuine benefits of various natural products.

1.1.1 Ayurveda and other botanicals as a source of lead molecules

Ayurveda, one of the ancient yet living sciences practiced successfully in India, Sri Lanka and other countries has a sound philosophical and experimental basis (Doiphode and Vijay, 2002). Pharmaceutical scientists are now experiencing

difficulty in identifying new lead structures, templates and scaffolds in the finite world of chemical diversity. A wide range of chronic and difficult-to-treat diseases like cancers, cardiovascular disease, diabetes, rheumatism and AIDS, all require new effective drugs (Patwardhan, 2005).

Ayurvedic medicines follow a 'reverse pharmacology' path, as in Ayurvedic medicine research, clinical experiences, observations, or available data become the starting point (Vaidya *et al.*, 2001). Ayurvedic knowledge and experiments can provide new functional leads to reduce time, money and toxicity, the three main hurdles of drug development. An Ayurvedic database could help researchers to start the search for drug discovery from well-tested and safe botanicals.

1.2 ESSENTIAL OILS

Essential oils, *olea antherea* or essences constitute the odorous principles of the plant in which they exist. They pre-exist in plants or are produced by the reaction of certain constituents when the tissues are brought in contact with water. They are called essential because they were once thought to be the essence of the plants (Remington and Osol, 1980) Essential oils are complex mixtures comprised of many single compounds and each constituent may be responsible for the characteristic beneficial or adverse effect (Buchbauer, 2000).

Depending on the plant family, essential oils may occur in different specialised structures such as glandular hairs (Lamiaceae), modified parenchyma cells (Piperaceae), oil-tubes called vitae (Umbelliferae) or in lysigenous or schizogenous passages (Pinaceae), (Kalodera *et al.*, 1998). Essential oils may accumulate in different organs of the plant (leaves, flowers, fruit, stem or roots) in a manner characteristic of the family of the individual plant (Strauss, 1973).

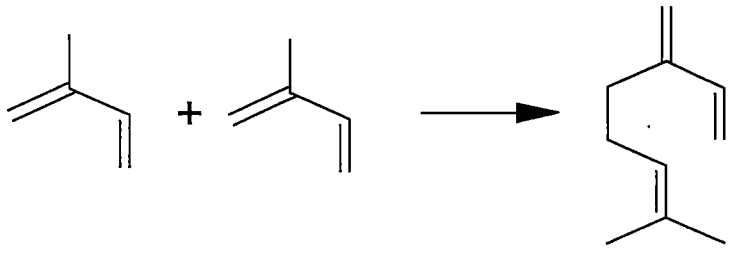
Each essential oil is made up of different chemical components, combined in different ways to create specific oils. The chemical compounds of essential oils are broken down into two main groups: hydrocarbons and oxygen based compounds. The oxygen based compounds are phenols (e.g. eugenol, carvacrol), esters (e.g. linalyl acetate, geranyl acetate), aldehydes (e.g. citral, geranial), ketones (e.g. thujone, pulegone), alcohols (e.g. linalool, geraniol) and oxides. The hydrocarbon portion is mainly monoterpene and sesquiterpene hydrocarbons. Brief classifications of the different classes of the terpenoids are summarized in Table 1.1.

Table 1.1 Classes of Terpenoids (Adapted from Mann *et al.*, 1994)

C_n	Name	Parent(s)	Occurrence in plants
C_5	Hemiterpenoid	IPP[a], DMAPP[b]	(Few) emissions, oils
C_{10}	Monoterpenoid	GPP[c]	Oils; petals
C_{15}	Sesquiterpenoid	FPP[d]	Oils, resin, petals
C_{20}	Diterpenoid	GGPP[e]	Oils; resins; heart-wood
C_{25}	Sesterterpenoid	GFPP[f]	Oils; resins, heart-wood
C_{30}	Triterpenoid	Squalene	Resin, heart-wood, leaf wax
C_{40}	Carotenoid	Phytoene	All green tissue; roots; petals
C_n (n=45 to 10^5)	Polyisoprenoid	GGPP[e]	Latex; leaf wax

[a]Isopentyl pyrophosphate [b]3,3- dimethylallyl pyrophosphate [c]geranyl pyrophosphate [d]2E,6E-farnesyl pyrophosphate [e]2E,6E,10E-geranylgeranyl pyrophosphate [f]2E,6E,10E,14E-geranylfarnesyl pyrophosphate

The lower terpenoids especially C_{10} and C_{15} compounds have been a subject of study since the dawn of modern chemistry (Table 1.1). Terpenoids are composed of linked C_5 units often called isoprene (2-methylbuta-1,3-diene) units. In general according to the isoprene rule, these isoprene units are linked in 'head-to-tail' fashion to form higher terpenoids (Fig. 1.1).



2 methylbuta-1,3 diene beta- myrcene

Figure 1.1 Linking of isoprene units

The isoprene rule was modified to the 'biogenic isoprene rule' which states that each terpenoid subgroup is derived from a single parent compound unique to that group. For example, sesquiterpenoids are all derived from FPP (Table 1) by a sequence of cyclizations, functionalizations, and rearrangements.

Terpenoid compounds have been separated by chromatographic techniques such as gas chromatography (GC), liquid chromatography (LC), thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Their structures have been determined by infrared (IR), ultraviolet (UV), nuclear magnetic resonance (NMR) spectroscopy and also mass spectrometry (MS). Commercial availability of radioisotopes of carbon and hydrogen led to an increase in the knowledge of structure types, synthesis, and the biosynthesis of terpenes (Mann *et al.*, 1994).

1.3 CUPRESSACEAE FAMILY

The family Cupressaceae (cypress pines) includes about 140 species from regions of moderate or warm climate in both hemispheres (Malizia *et al.*, 2000). *Callitris* is a genus of coniferous trees in the Cupressaceae family.

1.3.1 Callitris

The word *Callitris* is derived from the Greek word *kallistos*, which means most beautiful (Dallimore and Jackson, 1966). There is an ambiguity regarding the number of *Callitris* species. Different authors state different figures ranging from 13-20 (Dallimore and Jackson, 1966; Evans 1989). Although two species of *Callitris*, *viz C. quadrivalis* and *C. articulate*, are present in North Africa, the rest are found in Australia.

Callitris spp, often called cypress pines, cover around 4 300 000 hectares of forest (Logan *et al.*, 1985). *Callitris* comes under the Kingdom Plantae, Division Pinophyta, Class Pinopsida, Order Pinacles, Family Cupressaceae, and Subfamily Callitroideae. *Callitris* spp. are evergreen bushes or trees with hard bark and short erect branches which divide into branchlets at the closely pressed, sheath-like bases, while the buds are hidden by the leaves (Dallimore and Jackson, 1966). *C. rhomboidea* and *C. oblonga* plants were the species used in this research study.

1.3.1.1 Callitris oblonga

Callitris oblonga (Rich. et A. Rich) commonly called South Esk pine (Fig 1.2) or dwarf cypress pine is an evergreen tree, native to New South Wales and Tasmania. It prefers light rocky soils in an open sunny position and is drought and frost resistant and very hardy. It grows to a height of 5 m with a spread of 2 m. The stem is erect and branching, with a dense conical crown; the leaves are grayish green; the male catkins are terminal and the female catkins are oval; the fruit are conical and 2.5 cm long with 6 thick valves; and propagation is by seed or by cuttings (Bodkin, 1986).

Figure 1.2 Young *C. oblonga* plants.

1.3.1.2 Callitris rhomboidea

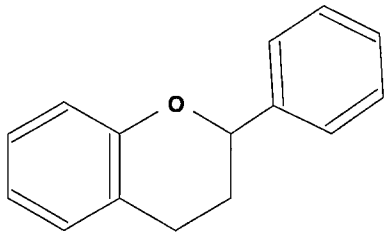
Callitris rhomboidea (R. Br. ex Rich) commonly called Oyster Bay pine (Fig 1.3) or Port Jackson pine, is an evergreen tree growing to a height of 12 m with a spread of 3 m. It is indigenous to all states of Australia except Western Australia. The tree prefers light, well-drained soils in a protected, semi-shaded position and is drought and frost resistant, and hardy. The stem is erect, with hard, furrowed bark, dense crown, weeping branches; leaves are short, fine, crowded, and bluish green, turning reddish bronze in winter. The male catkins are ovate solitary and terminal and the female catkins occur in clusters at the base of the branches; the cones are globular and 2 cm across, occurring in small clusters (Bodkin, 1986).

Figure 1.3 *C. rhomboidea* tree

1.3.2 Secondary metabolites from Callitris species

1.3.2.1 Flavonoids

Flavonoids are described by this common structure.



[1] Flavonoid

From the ethanolic leaf extract of *C. glauca*, kaempferol-5-O-rhamnoside was isolated along with 3,4,5,7,8-pentahydroxyflavone (also called hypoletin), amentoflavone and sesquoiaflavone (Ansari *et al.*, 1981). Further investigation of the same species by Khan and Ansari, (1987) detected the presence of myricetin-7-arabinoside, a flavonoid glycoside, along with quercetin, kaempferol, galangin, and shikimic acid.

On examining the ethanolic extracts of leafy twigs of *C. columellaris*, *C. endlicheri*, *C. preissii*, *C. canescens* and *C. macleayana*, amentoflavones such as 4-methylamentoflavone, and, from *C. canescens*, 4,7-dimethylamentoflavone were isolated (Gadek and Quinn, 1982). Chemical investigations conducted by the same authors in 1983, on the leaf extracts of *C. columellaris*, *C. muelleri* and *C. robusta*, found amentoflavone, cupressuflavone, and hinokiflavone (Gadek and Quinn, 1983). Hinokiflavone was previously detected from leaf extract of *C. glauca* and *C. rhomboidea* and was confirmed by TLC analysis (Ansari *et al.*, 1981; Khan *et al.*, 1979). In addition to the above-said flavonoids, a new flavonoid, mono-O-

methylamentoflavone, was isolated from the leaf extract of *C. rhomboidea* (Khan *et al.*, 1979).

1.3.2.2 Lignans

Lignans are one of the major classes of phytoestrogens, which are estrogen-like chemicals. Podophyllotoxin, a lignan, is the pharmacological base for the etoposide used in chemotherapy for malignancies such as Ewing's sarcoma, lung cancer, testicular cancer, lymphoma, non-lymphocytic leukemia, and glioblastoma multiforme. The first reported study on the occurrence of podophyllotoxin in *Callitris* spp. was done by Fitzgerald *et al.*, (1957). Kier *et al.*, (1963) reported that needles of *C. drummondi* produced 1.4% podophyllotoxin on a dry weight basis, and this lignan was entirely present in the β-D-glucosidic form. Later Van Uden *et al.*, (1990) found that needles of *C. drummondi* contained 1.5% podophyllotoxin calculated on a dry weight basis, however only 32% was present in the glycosidic form. Biotechnological production of podophyllotoxin was undertaken from the needles of *C. drummondi* and it was found that podophyllotoxin accumulated from 0-0.1% on a dry weight basis in the callus culture (Van Uden, 1993).

1.3.2.3 Tannins

Tannins are astringent, bitter plant polyphenols that either bind and precipitate or shrink proteins. Studies done by Coombs and Dettman, (1914) state that although less used by Australian tanners, pine barks have great potential as a tanning material. Bark from *C. calcarata* was found to contain 23.5% on dry weight basis of tannins (Coombs and Dettman, 1914). This tannin gave leather water-resistant properties and it was considered that it would doubtlessly give good sole leather when combined with wattle bark, which would be better than a straight wattle tannage. A straight pine

tannage produces a red leather, harsh but water-resistant with good weight returns (Coombs and Dettman, 1914; Coombs, 1919). Reinvestigation by Coombs *et al.*, (1925), found that tannin content is same at all heights on the tree (*C. calcarata*) average tannin content being 20-25% and maximum was 37%. It was greatest in small, well-grown trees, and in the outside of the inner zone of bark. It was later found that this property of the tan was due to the presence of a catechol tan with abnormally high acidity, excellent solubility and a high ratio of tans to non-tans (Purss and Anderson, 1947).

Reaction of tannins with formaldehyde gave resins that could be used as adhesives for wood. Adhesives prepared from tannins of *C. calcarata* gave bonds with strength somewhat lower than those obtained from typical common adhesives, but had better water resistance than the cold-set urea-formaldehyde adhesives (Dalton, 1950).

The trunk of several *Callitris* species yields a yellow resin and gum, which is marketed as sandarac resin or gum (Fitzgerald *et al.*, 1957; Carman and Deeth, 1967; Gough, 1968) and is used as a pharmaceutical aid in ointments and plasters as well as a tablet coating that dissolves in the intestine (Barr *et al.*, 1988).

1.3.2.3 Terpenoids

The work done by Baker and Smith on the steam distilled (SD) oil from leaves and stem of *Callitris* plants in 1910, was the first chemical extraction work reported in the family. Although they succeeded in identifying a few mono- and sesqui-terpenes their work was restricted due to poor oil yields. Hydrodistillation of the leaves of *C. rhomboidea* gave an oil containing geranyl acetate (60%) and free geraniol (14%) in addition to *d*-pinene (α-pinene) and *l*-pinene (β-pinene), limonene and dipentene (Smith , 1912). *C. rhomboidea* plant leaves from India were steam distilled to produce

an oil in the yield of 0.17% containing 17% esters and 50% terpenes (Rao *et al.*, 1925). The absence of the present day's common analytical instruments, like GC-MS, seriously crippled the pine oil research investigators prior to the 1980's (Brophy *et al.*, 2007).

Callitris spp. heartwoods are noted for their decay and termite resistance (Smith, 1912; Rudman, 1963). It was understood that decay resistance varied between different cypress pine trees and also within individual trees. However the studies done by Rudman, (1963) concluded that faster growing trees had lower decay resistance. *C. columellaris* heart wood was found to contain citronellic acid, together with α-, β- and γ-eudesmol and cryptomeridol (Rudman and Gay, 1964). Rudman (1965) tested heartwood and bark extracts of *Callitris* plants against moulds such as *Certhidea olivacea* and *Lentinus lepideus* and concluded that wood extracts did not control the growth of a broad spectrum of fungal species, but may be quite specific in their antifungal activity.

In addition to sandaracopimaric acid, the diterpenoid 4-epidehydroabietic acid, previously named as callitrisic acid, has been obtained from the sandarac resin from this pine (Carman and Deeth, 1967; Gough, 1968; Mori and Matsui, 1968; Chuah and Ward, 1969; Huffman, 1970).

A new diterpene acid δ13(17)-communic acid was isolated from *C. columellaris* (Atkinson and Crow, 1970). Yazaki and Hillis (1977) isolated five unidentified sesquiterpene lactone compounds from *C. columellaris* heartwood. One year later Brecknell and Carman (1978), isolated and identified six sesquiterpene lactones, the elemanolide callitrin, the eudesmolides callitrisin and dihydrocallitrisin, the guaianolides dihyrocolumellarin and columellarin, and a germacranolide $C_{15}H_{22}O_2$,

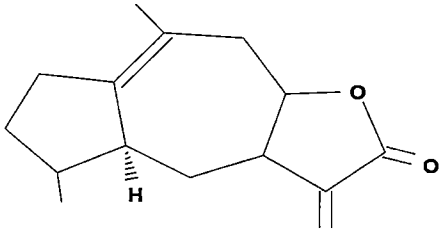
from the heartwood of *C. columellaris*. These compounds were the first sesquiterpene lactones identified from a member of the Cupressaceae family, and their structures have been reported to be interesting because of the unusual stereochemistry of the lactone ring. These lactones share a novel *cis*-fused ring in which the C7-C11 bond has an axial orientation to a cyclohexane ring (Brecknell and Carman, 1979; Godfrey and Schultz, 1979).

A chemosystematic study of *Callitris* spp. has been performed by Adams and Simmons (1987) using the volatile oils of *C. columellaris*, *C. preissii*, *C. verrucosa*, *C. endlicheri*, and *C. rhomboidea*. The monoterpenes present in the oils of all species were camphene, terpinolene, α-pinene, terpinen-4-ol, *α*-pinene, myrcene, limonene, geranyl acetate, and geraniol. The composition of the oils of *C. columellaris* and *C. preissi* was quite similar with the presence of *α*-pinene and myrcene as the major compounds. It was stated that the volatile oil composition was characteristic of each species.

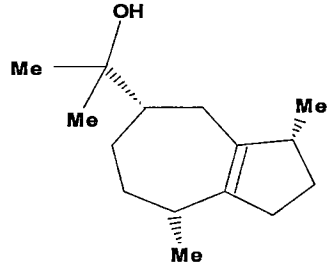
The first authenticated work on the composition of a complete list of SD oils obtained from the foliage of *Callitris* spp. was published in 2007 by Brophy and his colleagues. They reported the presence of a predominant amount of monoterpenes from the *Callitris* spp.

Callitris spp. timbers (*C. glaucophylla*, *C. endlicheri* and to a lesser extent *C. macleayana* and *C. columellaris*) are known for their resistance against termite attack. Studies done by Watanabe and coworkers in 2005 analysed the methanolic stem extracts of *C. galucophylla*. They identified that anti-termite activity of the timbers was mainly due to the presence of columellarin [**2**] and an undescribed sesquiterpene lactone compound.

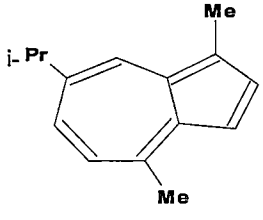
The decoction of *C. intratropica* bark in water was used by Australian aboriginal people as a wash for abdominal cramps (Barr *et al.*, 1988). Later steam distilling the wood of *C. intratropica* gave blue oil. The blue colour of the SD oil was due to the presence of guaiol [**3**] and guaiazulene [**4**] and chamazulene [**5**]; classified as azulenes (Doimo, 2001).



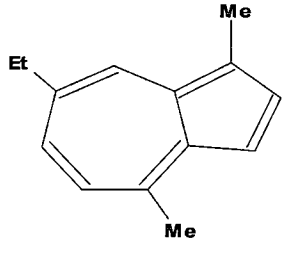
[**2**] Columellarin



[**3**] Guaiol



[**4**] Guaiazuline



[**5**] Chamazulene

The current work is a continuation of the research work done during the period of 2005-2006 for a Master of Pharmaceutical Science degree (Philip, 2006). We previously identified the presence of essential oils in the foliage and roots of *C. rhomboidea* and *C. oblonga* plants. Our findings revealed that about 50% of the total plant oils were present in the roots. The presence of essential oils in the roots of *C. rhomboidea* and *C. oblonga* has not been previously described.

Steam distilled as well as supercritical fluid (SFE) extracted oil sample were analysed by GC-MS. It was found that difference in extraction method had little effect on the composition of the oils. Although the presence of total percentage of monoterpenes was low when compared to sesqui-terpenes, one monoterpene compound $C_{11}H_{16}O_2$ (kovats indices KI 1223) needs a specific mention as it was present in common in both root oils. The major sesquiterpene compound reported from *C. rhomboidea* root oil was longiborneol while an unknown compound (UC) with KI 1984 was the major compound isolated from *C. oblonga* oil.

The *C. rhomboidea* foliage SD oil contained mainly citronellyl, neryl and geranyl acetate and not surprisingly geraniol, nerol and citronellol. *C. oblonga* foliage oils were rich in α-pinene, isopulegol, α-terpineol, and surprisingly the same sesquiterpene compound found in the roots UC (KI 1994).

A narrow range of anti-microbial, anti-fungal, anti-tumour and anti-viral testing was performed on the neat oils as well the methanolic extracts of *C. rhomboidea* and *C. oblonga* leaves. The tests were performed by the Chemistry Department, University of Canterbury, New Zealand. Methanolic extracts of the leaves were found to possess some activity against herpes simplex virus (HSV) and p388 murine leukaemia cells.

We hypothesised that the roots of the plants may release the volatile constituents into the soil and surrounding areas to produce a protective antimicrobial and allelopathic activity. The objectives of the current study were to:

- Further characterise the composition of essential oils from different plant parts of *C. rhomboidea* and *C. oblonga.*
- To identify some of the major unknown constituents in the root oil.

- To investigate the composition of the solvent extracts.
- To investigate the biological activities of the *C. rhomboidea* and *C. oblonga* SD oils and extracts against a range of bacteria and fungi.
- To investigate the release of essential oils from *C. rhomboidea* roots *in situ.*
- To investigate the allelopathic activities of *C. rhomboidea* and *C. oblonga* root and leaf oils.

CHAPTER 2

Chemical Composition of the Essentials Oils and Solvent Extracts Obtained from Different Organs of *Callitris rhomboidea* and *Callitris oblonga.*

The objective of the study was to describe and compare steam distilled (SD) oils and organic extract composition of leaf, bark, fruits, and roots of *C. rhomboidea* and *C. oblonga.*

2.1 GENERAL EXPERIMENTAL

2.1.1 Reagents used

The solvents used for various experiments were dichloromethane (DCM) laboratory grade reagent (LR) (BDH, Melbourne, Australia); methanol LR (> 98%) (Ajax Finechem, Sydney, Australia); hexanes analytical grade (AR) (BDH, Melbourne, Australia) and petroleum spirit (BDH, Melbourne, Australia). Solvents were freshly redistilled before use. Anhydrous magnesium sulphate (AR) (Sigma-Aldrich, Sydney, Australia) was used as a drying agent. Normal hydrocarbons (C_9-C_{22}) from Sigma-Aldrich, Sydney, Australia were used as standards for the determination of Kovats indices.

2.1.2 Instruments

2.1.2.1 Steam distillation apparatus

A Clevenger-type apparatus was used for steam distillation (SD). The apparatus consisted of a round bottom flask (2 l), combined with condenser and a side arm tube with stopcock. Preweighed plant material was added to the round bottom flask. The

round bottom flask was kept in a suitably sized heating mantle. The amount of heat could be adjusted by manual control.

2.1.2.2 Gas Chromatography- Mass Spectrometry (GC-MS)

Oil samples and solvent extracts diluted in hexane (5 mg/ml), were analysed by GC-MS using a Varian 3800 GC connected to a Varian 1200 triple quadrupole mass spectrometer (MS). The column was a 30 m x 0.25 mm diameter, 0.25 micron film VF5-ms, Factor Four from Varian. A Varian 1177 injector was used in split mode. The carrier gas was helium at a flow rate of 1.2 ml/minute in constant flow mode; the injection temperature was 220 °C. Oven temperature profile was 60 °C for one minute then to 210 °C at 6 °C per minute then programmed to 270 °C at 25 °C per minute with a 5 minute hold at 270 °C. Samples (1 µl) were injected split (20:1). The ion source temperature was 220 °C and the transfer line was held at 290 °C. The range from *m/z* 35 to 350 was scanned three times every second. The data was processed using the Varian Star Version 4 programme. All the hardware and software was supplied by Varian Inc (Melbourne, Australia).

2.1.2.3 Gas Chromatography

GC analysis of the essential oils was performed using a Varian 3800 gas chromatograph equipped with a flame ionisation detector (FID), and a data handling system (Varian Star Version 4). The same VF5-ms fused silica column used for GC-MS analysis was used for GC-FID analyses. The carrier gas was nitrogen at a flow rate of 0.4 ml/minute in constant flow mode. The injection temperature was 220 °C. Samples (1 µl) were injected split (20:1). Oven temperature profile was 60 °C for one minute then to 210 °C at 6 °C per minute then programmed to 270 °C at 25 °C per minute with a 5 minute hold at 270 °C.

2.1.2.4 Rotary evaporator

A rotary evaporator (Rotavapor-R manufactured by Buchi, Postfach, Switzerland) was used for removing the excess solvents from the solutions.

2.1.2.5 Mass Spectrometer

Accurate mass measurement was undertaken on a Kratos concept ISQ mass spectrometer (Kratos Analytical, Manchester, UK) in the electron ionization (EI) mode.

2.1.3 Plant preparation

Plant materials for extraction were obtained from Pulchella Nursery, Buckland, Tasmania and Plants of Tasmania Nursery, Hall Street, Ridgeway, Tasmania. Bulk collections of *C. rhomboidea* aerial parts were made from trees growing in front of the Tasmanian Herbarium, University of Tasmania, Sandy Bay and from the garden of C. Narkowicz, Scott Street, Bellerive.

The plants bought from the nurseries (15 cm and 25 cm pots), were uprooted from the pots and cleaned with tap water to remove potting mixture from the roots. The roots were dried with paper towel. Plant parts were separated as roots, leaves, and bark from each plant bought from plant nurseries. Wood samples and fruit samples were collected from Bellerive. The plant parts were weighed prior to extraction.

2.2 EXTRACTION TECHNIQUES

2.2.1 Steam Distillation

Water was added to the round bottom flask to cover all the plant material. The round bottom flask was attached to the Clevenger-type apparatus. Distillation was carried out for 3 hours with distilled water in the side arm of the apparatus. The oil that

accumulated over the water was transferred with a Pasteur pipette, dried over anhydrous sodium sulphate then transferred into a preweighed vial and stored at a temperature of less than 4 °C until analysis by GC-MS or biological screening. All oils were analysed by GC-MS within 24 hours of their production.

2.2.2 Solvent extraction

Solvent extraction was performed on roots, leaves, stem, bark and fruits of *C. rhomboidea* and *C. oblonga* plants, using organic solvents. Plant parts were soaked in solvents for one day. Each plant part was extracted with each solvent at least thrice. Initial extraction was performed with petroleum spirit, followed by DCM and then methanol. Extracts using each solvent were combined separately, concentrated and evaporated to dryness using a rotary evaporator. Each extract was weighed and transferred into a vial.

2.3 INTERPRETATION OF TERPENOIDS BY GC-MS

The identification of the extract and oil components was based on the comparison of mass spectra with the mass spectra in the National Institute of Standards and Technology (NIST) database, together with a comprehensive 'in-house' library of spectra accumulated over the years at the Central Science Laboratory, University of Tasmania. This includes many literature spectra and also spectra from various institutes such as the Commonwealth Scientific and Industrial Research Organization (CSIRO), and L'Institute National de la Recherche Agronomique (INRA). Further authentication of the components was assured by comparing the retention indices with published Kovats indices (Davies, 1990). Kovats indices (KI) were calculated using an *n*-alkane series (C_9-C_{25}) for which the retention time of each alkane was determined under the same GC conditions as for the samples.

2.4 RESULTS AND DISCUSSION

Oil yields from different samples of the *Callitris* spp. are reported in Table 2.1.

Table 2.1 Essential oil yields obtained from different parts of two *Callitris* spp.

Species and plant part (Plant source)	Mass of Plant Material (g)	Oil weight (g)	% Yield w/w
C. rhomboidea root (Buckland)	1100	3 1	0.28
C. rhomboidea fruit (Bellerive)	467	1 1	0 23
C rhomboidea bark (Bellerive)	265	0.7	0.27
C. rhomboidea leaf (Bellerive)	620	0 8	0.29
C oblonga root (Ridgeway)	260	0 5	0.20
C. oblonga fruit (Ridgeway)	465	1.1	0.24
C. oblonga leaf (Ridgeway)	324	0 6	0.19

In general, on comparing the yield of two *Callitris* spp., higher yields of oil were obtained from *C. rhomboidea*. The mean oil yield obtained from different organs of *C. rhomboidea* plants was 0.26±0.3% (mean±SD) while the yield obtained from different organs of *C. oblonga* was 0.22±0.2%.

In general the yield obtained from C. *rhomboidea* foliage was within the range reported by Brophy and colleagues (2007). They reported yields within the range 0.1-1.0%, for plants collected from different parts of Tasmania while plants collected from other parts of Australia (Vic, SA, NSW, Qld) yielded 0.1-0.5%. By comparison Baker and Smith (1910) distilled 280 Kg of foliage from Australia to obtain 0.112 kg of oil, a yield of 0.04%, which was below the yield obtained from our experiments and yields obtained by Brophy and colleagues. The yield of foliage oil obtained from *C. oblonga* was 0.19% which was less in yield when compared with *C. rhomboidea* foliage oil (0.29%). Baker and Smith reported the oil yield of *C. oblonga* foliage to be 0.06% while Brophy and colleagues reported the yields of foliage oil of *C. oblonga* ssp. (*oblonga, corangensis, parva*) to range from 0.1-0.3%. The variation in oil yields

could be due to the influence of climatic conditions, seasonal variation (Boussaada and Chemli, 2007) extraction method (Babu and Kaul, 2005), geographical variation (Viljoen *et al.*, 1995) or inter-tree variation (Kamdem and Hanover, 1993).

On direct comparison of yields between two *Callitris* spp, the oils obtained from *C. rhomboidea* leaf and roots were higher than *C. oblonga*. The oil yield (w/w) from the *C. rhomboidea* leaf was 0.29% and root yielded 0.28%; while *C. oblonga* leaf yielded 0.19% and roots yielded 0.20%. *C. oblonga* bark was not included in the study because there was not sufficient plant material.

Oils obtained from other plant parts (roots, bark, and fruit) were studied for the first time. The most interesting characteristic was the presence of the same amount of oil stored in the roots as in the leaves of both the *Callitris* spp.

2.4.1 Essential oil Compositions

2.4.1.1 Root oils

Steam distillation of the roots of *C. rhomboidea* yielded a light brown oil. The GC-MS analysis of the oils performed on a nonpolar column revealed the presence of a number of terpenoids. The volatile composition of the oils obtained from roots, leaf, bark and fruit of *Callitris* spp. are given in Table 2.2 and Table 2.3. Table 2.2 details the relative amount of individual components of each oil expressed as peak area % relative to total ion current (TIC) of the whole oil. Table 2.3 details the percentage composition of each oil computed from the GC peak areas determined by FID, which were calculated as the mean values of two injections of each oil sample, by means of the internal normalisation method. The mass spectra of the unknown compounds are reported in Appendix 2. Diterpenes, which were considered beyond the scope of our study, were detected but are not included in the tables.

Thirty-six compounds were identified from the root oil of *C. rhomboidea*, accounting for about 85% of the total essential oils (Table 2.2). Monoterpenoids constituted around 11% of the total oil. The major monoterpenoids identified were tricyclene (1%), and camphene (1%). The main bulk of constituents (74%) were sesquiterpenes, and the TIC percentages of the most abundant constituents were longiborneol (23%), longifolene (5%), thujopsene (3%), α-chamigrene (2%), β-calacorene (2%), spathulenol (2%) and guaiol (1%).

Due to the complexity of the oil and the absence of descriptions of root oils from this family, identification of several compounds was very difficult. The major monoterpenoid unidentified was unknown compound 1 (UC1) accounting for about 6% of the oil. Accurate mass measurement of UC1 was undertaken and this was found to be 180.11456 amu corresponding to $C_{11}H_{16}O_2$. The mass spectrum (MS) of the compound contained the following peaks *m/z*(%): 180(29), 148(44), 121(100), 120(37), 119(28), 105(81), 93(67), 91(64), 88(67), 79(42), 77(47).

Table 2.2 Composition of *Callitris* spp. essential oils obtained from different organs of the plants as determined by GC-MS (percentage of total ion current). Peaks are listed in elution order from the VF5-ms column.

Compound	KI	*Callitris rhomboidea* root	*Callitris rhomboidea* fruit	*Callitris rhomboidea* bark	*Callitris rhomboidea* leaf	*Callitris oblonga* root	*Callitris oblonga* fruit	*Callitris oblonga* leaf
Tricyclene	925	1.2	0 3	0.4	0.2		0.2	0 2
α-Pinene	935	0.5	83 8	56.5	42 2		73 9	45.0
α-Fenchene	949			0 3				
Camphene	953	1.8	0 7	1 0	0.3		0 4	0.3
Mentha 1,4,8- triene	960			1 9				
Sabinene	966		0.1					
β-Pinene	974		2 3	5.7	1.1		1.2	0 3
Myrcene	988		1 5	3.2	1.3		0.6	1.0
δ-3-Carene	1009	0.2	0.5	5.0	0.7		0.1	0 2
Cymene	1023			0 7	0.1		0.4	0 3
β-Phellandrene	1030		0.6	1.3	0.2			
Limonene	1032	0 2	1.2	0.9	1.0		7.2	1.7
1,8-Cineole	1037	0.1		0 1				0.8
Cymenene	1082			1 2			0 3	
Terpinolene	1088	0.2	0.3	0.7	0 1			0 4
Cymenene	1090		0.3	1 8				
Linalool	1097				0.5			
cis-Rose oxide	1111							0 4
Fenchol	1125	0.3	0.2	0.1		0 3		
α-Campholene	1131		0.3	0 3	0.2		0.5	0 8
trans-Pinocarveol	1146		0.1	0 6	0.2		0.3	0 4
Verbenol	1149						0 4	
Citronellal	1150				1 1			
Isopulegol	1153							6.0
Camphor	1154		0 2					
Pulegol	1165							2.3
Pinocarvone	1169				0.1		0.3	0.3
Isoborneol	1170	0.2				0.2		
α-Phellandrene-8-ol	1177		0 8					
Borneol	1179	0.3		0 4		0.2		0.7
Cymenene	1082			1 2			0 3	
3-Pinanone	1185			0 1				
Terpinene-4-ol	1186			0.3				0.4
p-Cymene-8-ol	1194			0 2			0 2	
α-Terpineol	1200	0.1	0 6	0.9	0.2	0.2	1.5	2 2
Myrtenal	1203		0.2		0.3			1 0
Verbenone	1215		0.2	1.0			0.5	1.0
Unknown compound 1	1223	5.9				9.2		0.8
Citronellol(+some nerol)	1226			0.1	5.3			
Citronellol	1232							7 2
Pulegone	1248							0.3
Geraniol	1253				6.3			
Carvone	1255						0.3	
Methyl citronellate	1262				1.1			

Table 2.2 continued .

Compound	KI	*Callitris rhomboidea* root	*Callitris rhomboidea* fruit	*Callitris rhomboidea* bark	*Callitris rhomboidea* leaf	*Callitris oblonga* root	*Callitris oblonga* fruit	*Callitris oblonga* leaf
trans-Carveol	1288						0 2	
Bornyl acetate	1289			0.1	0.1		0.1	0.6
Unknown compound 2	1303					0 8		
Methyl geranate	1315			0.1				
Unknown compound 3	1335							0 7
Carveyl acetate	1336				1.4			
Citronellyl acetate	1349		0.3	0.3	6.7		0.1	
Neryl acetate	1358		0.2	0 2	5.5			
α-Longipinene	1359	0.3						
Unknown compound 4	1365							1.7
Cyclosativene	1376	2 8				1.4		
Geranyl acetate	1378		0.1	0.3	11 8			
Unknown compound 5	1386	1.2				0 4		
β-Elemene	1387					1.1		
Sativene	1399	0.3				0 1		
Longifolene	1419	4.9				2 9		
Isosativene	1423	0 5				0.2		
β-Cedrene	1424	0.6	0 2					
β-Cubebene	1425	0.5						
Unknown compound 6	1426							3 7
Citronellyl propionate	1441							0.3
Thujopsene	1444	3.1	1.2	1.2	2.2	1.0	0 6	0.4
Unknown compound 7	1461	4 7	2 2	2 5	3 5	2.2	1.8	0.4
Geranyl propionate	1468							0.1
β-Guanine	1478					0.8		
β-Ionone	1480					0.2		
γ-Muurolene	1481	0 4						
Germacrene D	1489	0.5			0 5			
β-Selinene	1498			0.1		1 5		
Bicyclogermacrene	1502						1.9	
α-Selinene	1504			0.1		1.4		
β-Bisabolene	1511							
Unknown compound 8	1515	7 3				0 7		
Unknown compound 9	1518					3.0		
Bornyl isovalerate*	1524		0 2	1 0				
δ-Cadinene	1525				0 1			
α-Chamigrene	1537	2 1						
Unknown compound 10	1553	2 9						
Unknown compound 11	1563	3.3				0 4		
Nerolidol	1564						0.4	
β-Calacorene	1571	2.0				1.5		
Spathulenol	1588	2.1	0.1		0 8		0 7	2 4
Geranyl isovalerate*	1591				0.3			
Guaiol	1604	1 3		1.1				
Globulol	1609							

Table 2 2 continued...

Compound	KI	*Callitris rhomboidea* root	*Callitris rhomboidea* fruit	*Callitris rhomboidea* bark	*Callitris rhomboidea* leaf	*Callitris oblonga* root	*Callitris oblonga* fruit	*Callitris oblonga* leaf
Longiborneol	1616	22.9				4.1		0.5
Unknown compound 12	1617	2.5						
Widdrol	1619		0 1	0.4		0.8	0.5	
Unknown compound 13	1622	2 5				0 7		
Unknown compound 14	1629	1 9						
γ-Eudesmol	1634			0.2				
β-Eudesmol	1647			0.3				
Unknown compound 15	1649	1.2				0.4		
Unknown compound 16	1659	1.0						
Unknown compound 17	1679	2 9						
Bulnesol	1675	0.5		0.2				
Unknown compound 18	1686	1 1				1 9		
Unknown compound 19	1700					0 6		
Farnesol isomer	1719				0 1			
Unknown compound 20	1732					1.2		
Unknown compound 21	1769					1 0		
cis-Methyl isocosticate*	1804	2 1				0.4		0 7
Unknown compound 22	1814					1.0		
trans-Methyl	1827							0.5
Farnesyl acetate	1834				0.1			
Unknown compound 23	1837					1 2		
Unknown compound 24	1850					3 4		
Unknown compound 25	1864					7 0		
Dihydrocolumellarin	1929					1 3		0.7
Columellarin	1984					29 5		6.9
Unknown compound 26	1992					0.6		
Unknown compound 27	1994					0 9		
Unknown compound 28	2004					1 2		
Unknown compound 29	2010					0.3		
Unknown compound 30	2015					1.2		
Unknown compound 31	2021					0.7		
Unknown compound 32	2030					1.0		
Total		86 4	97.8	93.8	96.9	89 9	94.6	93.6

*tentatively identified

Mass spectral data for unknown compounds *m/z* (%): **Unknown compound 1** 180(29), 148(44), 121(100),120(37), 119(28), 105(81), 93(67), 91(64), 88(67), 79(42), 77(47) **Unknown compound 2** 180(4), 165(9), 149(13), 138(20), 137(60), 136(33), 121(26), 105(100), 93(63), 91(71), 79(40), 77(56), 59(35) **Unknown compound 3** 134(27), 119(51), 105(29), 93(51), 79(43), 77(42), 43(100), 41(28), 39(20) **Unknown compound 4** 136(20), 121(20), 107(19), 94(15), 93(32), 84(12), 81(42), m79(19), 67(11), 57(100), 41(19) **Unknown compound 5** 204(6), 189(5), 175(4), 162(21), 161(100), 134(14), 133(15), 121(45), 119(20), 107(18), 105(21), 93(24), 91(20), 79(14) **Unknown compound 6** 134(35), 119(64),

105(41), 93(62), 92(100), 91(79), 84(21), 79(29), 77(28), 57(68), 41(19) **Unknown compound 7** 207(2), 189(10), 161(6), 133(8), 121(7), 119(11), 111(34), 109(24), 108(83), 107(21), 96(92), 95(64), 94(54), 93(100), 91(33), 81(61), 79(39), 77(22), 55(24), 41(21) **Unknown compound 8** 204(5), 189(5), 161(19), 105(41), 93(62), 92(100), 91(79), 84(21), 79(29), 77(28), 57(68), 41(19) **Unknown compound 7** 207(2), 189(10), 161(6), 133(8), 121(7), 119(11), 111(34), 109(24), 108(83), 107(21), 96(92), 95(64), 94(54), 93(100), 91(33), 81(61), 79(39), 77(22), 55(24), 41(21) **Unknown compound 8** 204(5), 189(5), 161(19), 147(4), 133(10), 119(78), 117(5), 107(50), 105(32), 93(100), 91(43), 79(34), 69(28), 55(25) **Unknown compound 9** 204(36), 189(60), 134(59), 133(42), 121(65), 119(44), 107(55), 105(39), 93(100), 91(44), 79(46), 68(50) **Unknown compound 10** 136(100), 121(72), 204(24), 93(22), 91(20), 107(19), 161(19), 105(18), 133(14), 119(12) 79(14), 41(13) **Unknown compound 11** 220(15), 200(13), 162(74), 157(72), 147(49), 133(31), 120(24), 119(49), 107(25), 106(47), 105(61), 102(76), 94(51), 91(46), 79(21), 59(100) **Unknown compound 12** 220(3), 206(1), 205(8), 204(15), 189(12), 187(19), 177(20), 176(100), 173(6), 162(11), 161(69), 159(30), 149(15), 147(16), 145(26), 135(36), 133(41), 121(36), 107(53), 105(63), 91(67) **Unknown compound 13** 222(14), 189(9), 164(10), 176(100), 161(59), 135(38), 133(42), 121(36), 119(40), 107(47), 105(58), 95(37), 93(37), 91(65) **Unknown compound 14** 220(6), 205(5), 187(12), 178(21), 177(18), 163(29), 162(66), 160(14), 159(100), 149(12), 147(37), 145(21), 137(13), 133(21), 131(30) **Unknown compound 15** 220(24), 204(18), 189(15), 187(20), 176(100), 161(59), 135(38), 133(45), 119(58), 107(58), 105(79), 93(51), 91(99), 79(52), 43(49) **Unknown compound 16** 220(24), 205(13), 187(25), 178(10), 161(59), 145(31), 133(38), 131(44), 121(58), 119(81), 105(910, 107(76), 94(100), 91(84), 79(54) **Unknown compound 17** 220(1), 207(20), 204(30), 163(18), 149(38), 135(16), 121(19), 119(11), 107(37), 95(67), 81(33), 59(100), 55(16) **Unknown compound 18** 222(5), 204(58), 189(58), 161(63), 135(88), 109(55), 95(67), 93(61), 81(96), 71(58), 67(57), 43(100) **Unknown compound 19** 218(16), 203(25), 200(10), 189(19), 189(19), 185(25), 175(23), 161(28), 158(44), 157(49), 149(50), 109(50), 95(58), 91(48), 81(40), 79(41), 59(100), 43(69), 41(43) **Unknown compound 20** 220(7), 205(5), 189(15), 163(15), 159(12), 145(14),105(50), 96(55), 95(100), 94(94), 93(78), 91(78), 81(54), 81(54), 79(53), 77(37), 55(40) **Unknown compound 21** 236(11), 221(34), 193(100), 175(31), 147(171), 121(58), 119(31), 105(44), 95(27), 93(31), 91(46), 79(31) **Unknown compound 22** 220(2), 175(30), 161(20), 147(27),133(31), 121(35), 107(36), 93(56), 91(63), 79(60), 68(100), 67(53), 41(35) **Unknown compound 23** 217(12), 158(84), 157(100), 156(36),155(13), 143(31), 142(84), 141(41), 129(11), 128(16), 115(22) **Unknown compound 24** 217(10), 172(19), 160(21), 145(40), 132(30), 121(56), 107(40), 105(43), 93(63), 91(100), 81(42), 79(49), 68(84),

67(47), 53(45), 41(40) **Unknown compound 25** 217(18), 199(13), 191(21), 171(22), 163(12), 161(16), 159(50), 149(12), 145(48), 135(26), 159(50), 145(48), 121(47), 107(43), 105(46), 93(68), 91(100), 79(50), 68(68), 67(52), 53(46) **Unknown compound 26** 232(20), 217(9), 207(10), 203(11), 171(13), 161(14), 160(18), 145(40), 143(83), 131(43), 119(82), 91(100), 81(51), 79(59), 77(57), 55(79), 41(87), 40(60), 39(59) **Unknown compound 27** 234(65), 233(34), 223(18), 206(58), 201(13), 191(35), 177(20), 149(65), 121(63), 71(35), 43(100), 41(37) **Unknown compound 28** 232(2), 232(32), 215(24), 197(28), 185(42), 169(34), 157(21), 143(50), 129(27), 119(100), 117(32), 115(21), 105(42), 91(69), 79(32), 77(41), 65(17), 53(27), 41(31) **Unknown compound 29** 232(6), 215(23), 212(4), 202(7), 201(14), 197(18), 187(11), 185(30), 169(33), 157(20), 145(35), 143 (46), 141(18), 133(11), 119(73), 105(55), 91(100), 77(40) **Unknown compound 30** 232(1), 231(6), 230(46), 215(57), 212(6), 207(6), 197(8), 185(24), 173(41), 159(40), 145(58), 108(25), 105(55), 91(100), 81(90), 77(67), 53(46), 41(63) **Unknown compound 31** 234(1), 232(17), 217(16), 204(100), 188(20), 159(23), 145(21), 121(30), 94(43), 79(42), 77(23), 53(23), 41(34) **Unknown compound 32** 234(3), 232(28), 217(26), 187(28), 121(100),105(29),91(34),79(28),41(29).

Table 2.3 Composition of *Callitris* spp. essential oils obtained from different organs of the plants as percentage composition of the oils computed from the GC peak areas (FID). Peaks are listed in elution order from the VF5-ms column.

Compound	KI	*Callitris rhomboidea* root	*Callitris rhomboidea* fruit	*Callitris rhomboidea* bark	*Callitris rhomboidea* leaf	*Callitris oblonga* root	*Callitris oblonga* leaf
Tricyclene	925	1.7	0.3	0.2	0 2		0.1
α-Pinene	935	0 7	83 7	73.3	43 1		19.3
α-Fenchene	949			0.6			
Camphene	953	2 7	0.7	0.5			0.2
Mentha 1,4,8- triene	960			1.0			
Sabinene	966		0.2				
β-Pinene	974		2 7	5.0	0.2		
Myrcene	988		1.7	2.8			0.9
δ-3-Carene	1009	0.2	0.6	0.4			0.1
Cymene	1023	0 3		0.6			0.4
β-Phellandrene	1030		0.4	0.4	1.0		
Limonene	1032		1.3	2.8	1 7		1.3
1,8 Cineole	1037			0.4	0.4		0 5
Terpinolene	1088		0.2	0.5	0.2		0.3
Cymenene	1090		0.2	0.4			
Linalool	1097			0.4			
cis-Rose oxide	1111			0.2			0.1
Fenchol	1125	0 3	0 2	0.3	0 1	0.3	
α-Campholene aldehyde	1131		0.2	0 4			0 9
trans-Pinocarveol	1146		0 1		0.5		1.4
Citronellal	1150			0.3			
Isopulegol	1153			0.2	0.1		4.0
Camphor	1154		0.1	0.2	0.1		
Pulegol	1165			0.4			0.1
Pinocarvone	1169				0 1		0.2
Isoborneol	1170	0.3				0.1	
α-Phellandrene-8-ol	1177				1 5		
Borneol	1179	0.1					0.4
Terpinene-4-ol	1186						0.4
p-Cymene-8-ol	1194				0.1		
α-Terpineol	1200	0 1	0 5		0.2		2 5
Myrtenal	1203		0.1	0 5	0 4		0 4
Verbenone	1215		0.1	0 1			0.5
Unknown compound 1	1223	6.1		0.3		11.8	0 6
Citronellol(+some nerol)	1226			0.1	5.1		
Citronellol	1232						7 8

Table 2 3 continued.

Compound	KI	*Callitris rhomboidea* root	*Callitris rhomboidea* fruit	*Callitris rhomboidea* bark	*Callitris rhomboidea* leaf	*Callitris oblonga* root	*Callitris oblonga* leaf
Pulegone	1248			1.1			0 4
Geraniol	1253				6 0		
Methyl citronellate	1262			0.1	1.1		
Bornyl acetate	1289				0.1		
Unknown compound 2	1303			0.1		0.5	
Unknown compound 3	1335						0 1
Carveyl acetate	1336				1.0		
Citronellyl acetate	1349		0 3		6.9		
Neryl acetate	1358		0 2		5.9		
α-Longipinene	1359	0.3					
Unknown compound 4	1365						1.9
Cyclosativene	1376	1.8				1 3	
Geranyl acetate	1378		0.2		10.9		
Unknown compound 5	1386	0.6				0.4	
β-Elemene	1387					1 0	
Sativene	1399	0.3					
Longifolene	1419	5.6				2.2	
Isosativene	1423	0.4				0.2	
β-Cubebene	1425	0.1					
Unknown compound 6	1426						3.2
Citronellyl propionate	1441						0.7
Thujopsene	1444	2 6	0 3		2 2	1 2	0.3
Unknown compound 7	1461	5.2	1 7		3.5	2 5	0.2
Geranyl propionate	1468						0.1
β-Guanine	1478					0.8	
β-Ionone	1480					0.1	
γ-Muurolene	1481	0.1					
Germacrene D	1489	0.2			0.5		
β-Selinene	1498					1.6	
α-Selinene	1504					1 4	
Unknown compound 8	1515	4.3					
Bornyl isovalerate*	1524		0 2	0.2			
δ-Cadinene	1525				0.2		
Unknown compound 10	1553	1 6				3 4	
Unknown compound 11	1563	1.6					
β-Calacorene	1571	1.7				1.5	
Spathulenol	1588	2.9	0.1		0 6		3.6

Table 2.3 continued...

Compound	KI	*Callitris rhomboidea* root	*Callitris rhomboidea* fruit	*Callitris rhomboidea* bark	*Callitris rhomboidea* leaf	*Callitris oblonga* root	*Callitris oblonga* leaf
Geranyl isovalerate*	1591				0 3		
Guaiol	1604			2.1			
Longiborneol	1616	32 6				4 5	1.9
Widdrol	1619		0.1	0.2		0.8	
Unknown compound 13	1622	1.3				0 7	
Unknown compound 14	1629	1.9					
Unknown compound 15	1649					0.4	
Unknown compound 18	1686					1.9	
Unknown compound 19	1700					0.6	
Unknown compound 20	1732					1.2	
Unknown compound 21	1769					0.9	1 5
cis-Methyl isocosticate*	1804					0 3	
Unknown compound 22	1814					0.9	
trans-Methyl isocosticate*	1827						1.3
Unknown compound 23	1837					1.0	
Unknown compound 24	1850					3.5	
Unknown compound 25	1864					8.0	
Dihydrocolumellarin	1929					1 5	1 0
Columellarin	1984					30 0	10 2
Unknown compound 26	1992					0.5	
Unknown compound 27	1994					0.9	
Unknown compound 28	2004					1.2	
Unknown compound 29	2010					0.1	
Unknown compound 30	2015					1.0	
Unknown compound 31	2021					0 7	
Unknown compound 32	2030					1.1	
Total		77.6	96 7	97.7	95 2	92 0	68.8

From first principle interpretations of the MS data, UC1 corresponds to a methyl ester of a monoterpene carboxylic acid. The methyl ester group (CH_3-COO-) has a mass of 59 amu. Fragmentation of the ester from the compound would give an ion with a base peak of mass 121 amu, as was the case for the above compound.

The sesquiterpenes were the most represented group in terms of the number of unidentified compounds (7). The unknown sesquiterpene compounds accounted for 29% of the total essential oil. The MS data of UC5, UC7 and UC9 correspond to sesquiterpene hydrocarbons with MW 204; while UC11-13 correspond to sesquiterpene alcohols which have a MW of 220. Unfortunately UC8 was a mixture of sesquiterpenes and it would be unwise to comment much further. Fig 2.3 shows typical GC-MS chromatograms of SD root oils from *C. rhomboidea* and *C. oblonga.*

Steam distillation of the roots of *C. oblonga* yielded light yellow oil. As shown in Table 2.2, 45 compounds were identified from the root oil corresponding to 90% of the total essential oil. Out of the total identified constituents monoterpenes accounted for 11% of the oil while sesquiterpenes constituted 79% of the oil. Unknown compound 1, previously detected in *C. rhomboidea* root oil, was the major monoterpenoid present (9.2 % of the total TIC of the oil) in *C. oblonga* roots. Present at a concentration below 1% was UC2, a monoterpenoid and a possible isomer of UC1. This compound eluted later from the column, and was undetected in *C. rhomboidea* root oil.

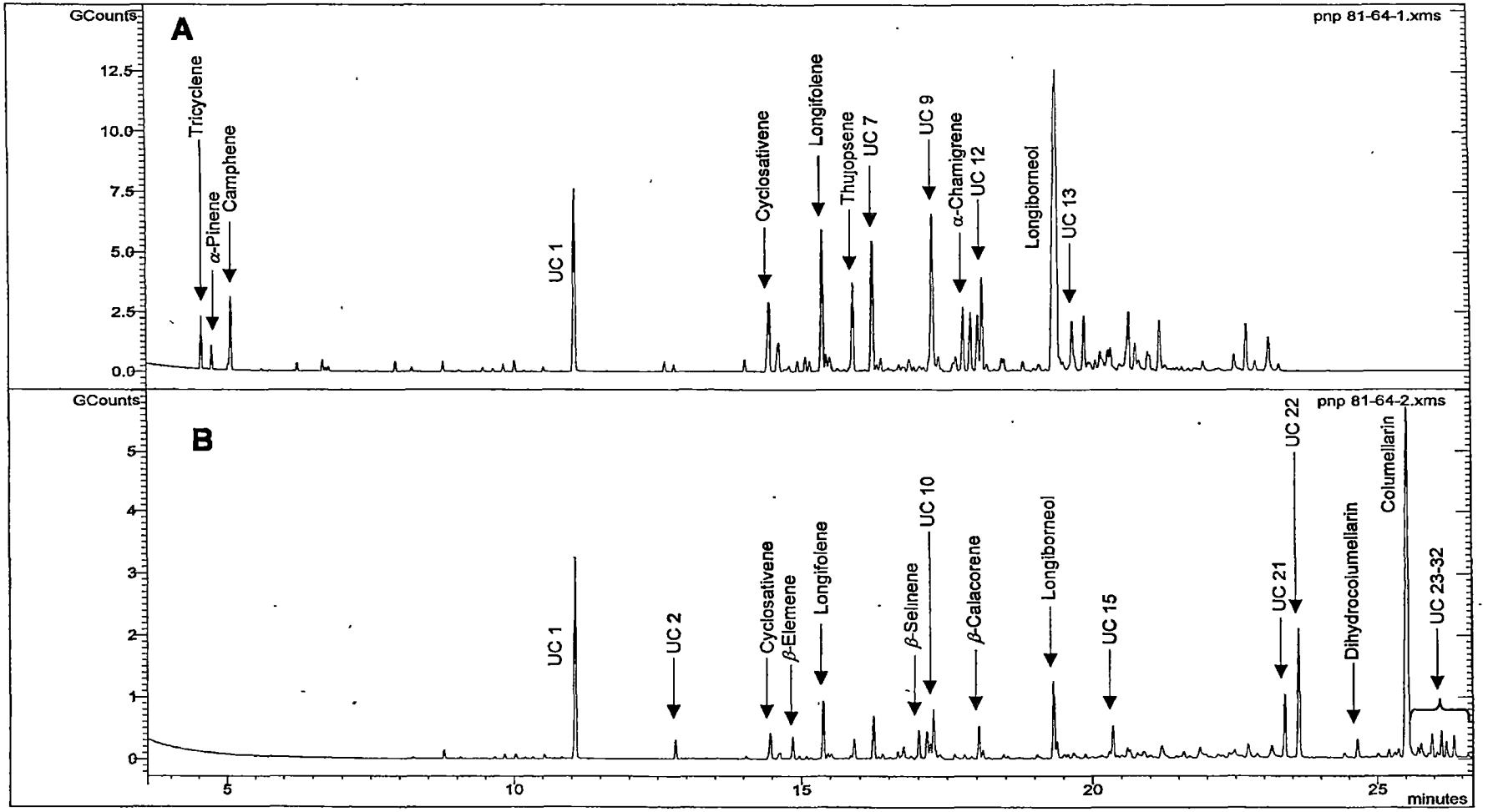


Figure 2.3 GC-MS chromatograms of steam distilled root oil of *C. rhomboidea* (A) and *C. oblonga* (B).

Out of the total 79% of the oil that consisted of sesquiterpenes, 46% of the total oil corresponded to late eluting γ-lactones with MW 232 and 234 while the remaining 33% consisted of sesquiterpene hydrocarbons. The major sesquiterpene hydrocarbons identified were longiborneol (4%), isosativene (3%), β-selinene (1.5%), cyclosativene (1.4%), and β-elemene (1%). The major γ-lactones identified were columellarin (30%) and dihydrocolumellarin (1.2%).

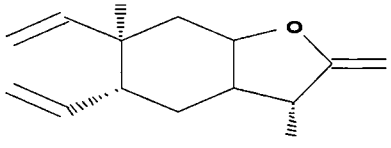
Unidentified γ-lactones (UC19-30) together constituted around 18.5% of the TIC of the oil, while sesquiterpene hydrocarbons (UC7 and UC9-18) accounted for about 12% of the oil. Methyl esters of isocostic acids [*m/z*(%): 248(43, M^+), 233(100), 217(10), 201(40), 173(60)] such as methyl-*cis*-isocosticate and methyl-*trans*-isocosticate were tentatively identified at concentrations below 0.5% in the oil (Doimo *et al.*, 2001).

Although the major monoterpenoid compound was UC1 in both the root oils (*C. rhomboidea* and *C. oblonga*) both oils were characterised by the presence of high amounts of sesquiterpenes. The most notable difference between the oils was the absence of sesquiterpene lactones in the former oil sample. Longiborneol constituted the major sesquiterpene in *C. rhomboidea* root oil while columellarin was the major constituent in *C. oblonga* root oil. This would suggest that both the species may have diverged, in evolutionary terms, a long time ago.

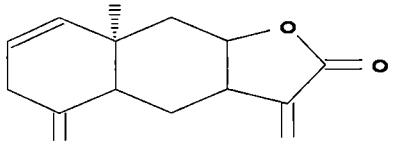
2.4.1.1.1 Identification of columellarin and dihyrocolumellarin

Sesquiterpene lactones from *Callitris* spp. were subjected to study since 1977 (Yazaki and Hillis). The first work done to isolate the sesquiterpene lactones from *C. columellaris* heartwood was done by Brecknell and Carman (1979). They were

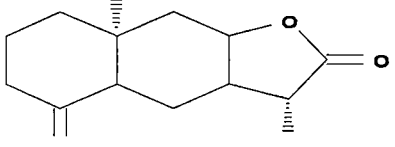
successful in isolating and describing five sesquiterpene lactones which were novel to the Cupressaceae family. The five sesquiterpenes isolated and described were callitrin **(5)**, callitrisin **(6)**, dihydrocallitrisin **(7)**, columellarin **(1)** and dihydrocolumellarin **(9)**. The structures of the compounds are given in Fig 2.4.



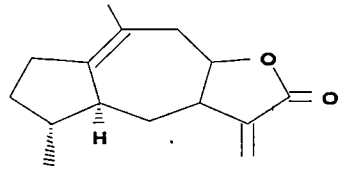
(5) Callitrin



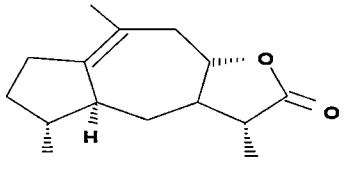
(6) Callitrisin



(7) Dihydrocallitrisin



(1) Columellarin



(9) Dihydrocolumellarin

Figure 2.4 Structures of γ-lactones isolated from *C. columellaris* heartwood

The MS of the sesquiterpene lactones were reported to be as follows: callitrin *m/z* (%) 234(19), 219(27), 206(12), 205(8), 193(39), 192(38), 173(16), 161(95), 160(100), 151(18), 145(64), 134(32), 133(240, 121(44), 119(78), 108(36), 107(23), 105(29), 95(32), 93(59), 91(24), 81(25), 79(21), 68(89), 55(40), 41(48); callitrisin 232(25),

217(3), 204(3), 203(3), 171(9), 159(80), 158(63), 145(20), 143(100), 131(40), 129(28), 119(92), 117(35), 105(53), 91(44), 79(40), 77(46), 67(21), 65(25), 55(41), 53(40), 51(19); columellarin 232(100), 217(11), 203(5), 199(7), 189(8), 175(6), 147(32), 136(28), 122(58), 107(73), 105(40), 91(44), 81(52), 69(20), 53(370, 41(44); dihydrocolumellarin 234(64), 219(14), 205(9), 191(5), 173(5), 161(16), 160(9), 147(12), 145(12), 138(27), 122(100), 107(68), 105(25), 93(31), 91(30), 81(28), 79(28), 55(26), 41(24) and dihydrocallitrisin 234(19), 219(8), 206(47), 161(21), 160(12), 145(26), 133(100), 121(17), 107(14), 105(24), 91(25), 79(22), 67(13), 55(21), 41(29).

The mass spectra of sesquiterpene lactones present in the *C. oblonga* SD root oil were compared with the mass spectra of the above said compounds for primary identification of compounds. Mass spectra of the compounds having KI, 1984 and 1929 closely resembled the mass spectra of columellarin and dihydrocolumellarin respectively. Kovats indices of these compounds were a good match with the KI published by Doimo and coworkers in 1999 using a fused silica column (BPX-5, SGE Inc, Victoria, Australia). Columellarin had a KI of 2005 and dihydrocolumellarin had a KI of 1948. Confirmation of these assignments was performed by running a reference sample of *C. intratropica* oil (kindly supplied by Aaron Pollack, Southern Cross University, Military Road, Lismore, NSW) with *C. oblonga* SD root oil. Fig 2.5 shows the GC-MS traces of the *C. oblonga* root oil and *C. intratropica* oil scanned for intensity of the ion at *m/z* 232 while Fig 2.6 shows the respective mass spectra.

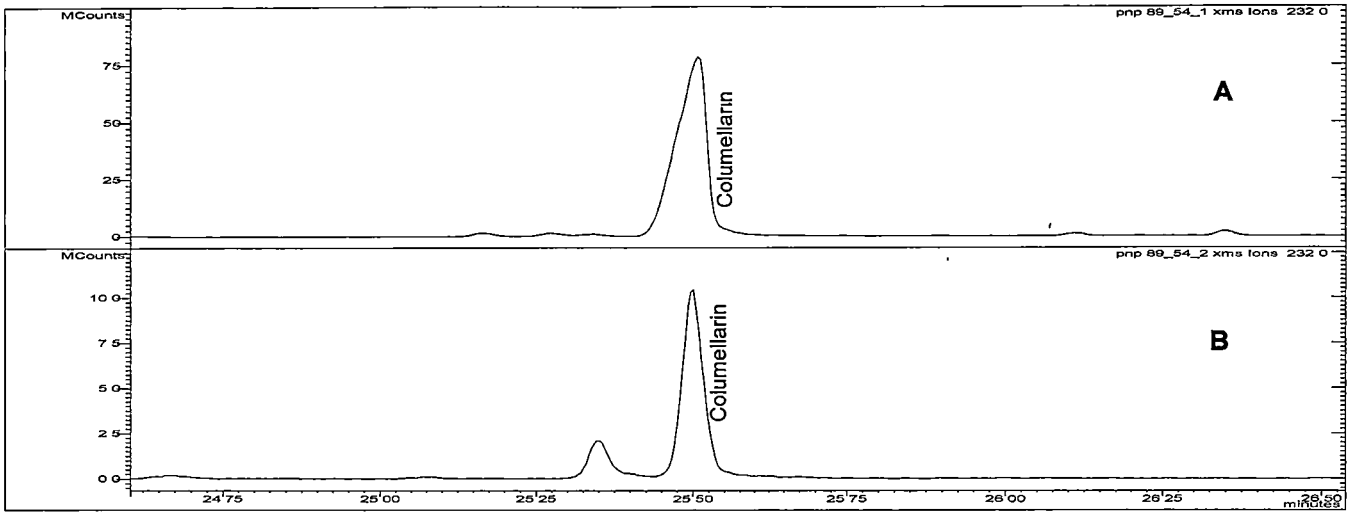


Figure 2.5 *m/z* 232 mass chromatograms, extracted from the full scan data of SD *C. oblonga* root oil (A) and *C. intratropica* stem SD oil (B).

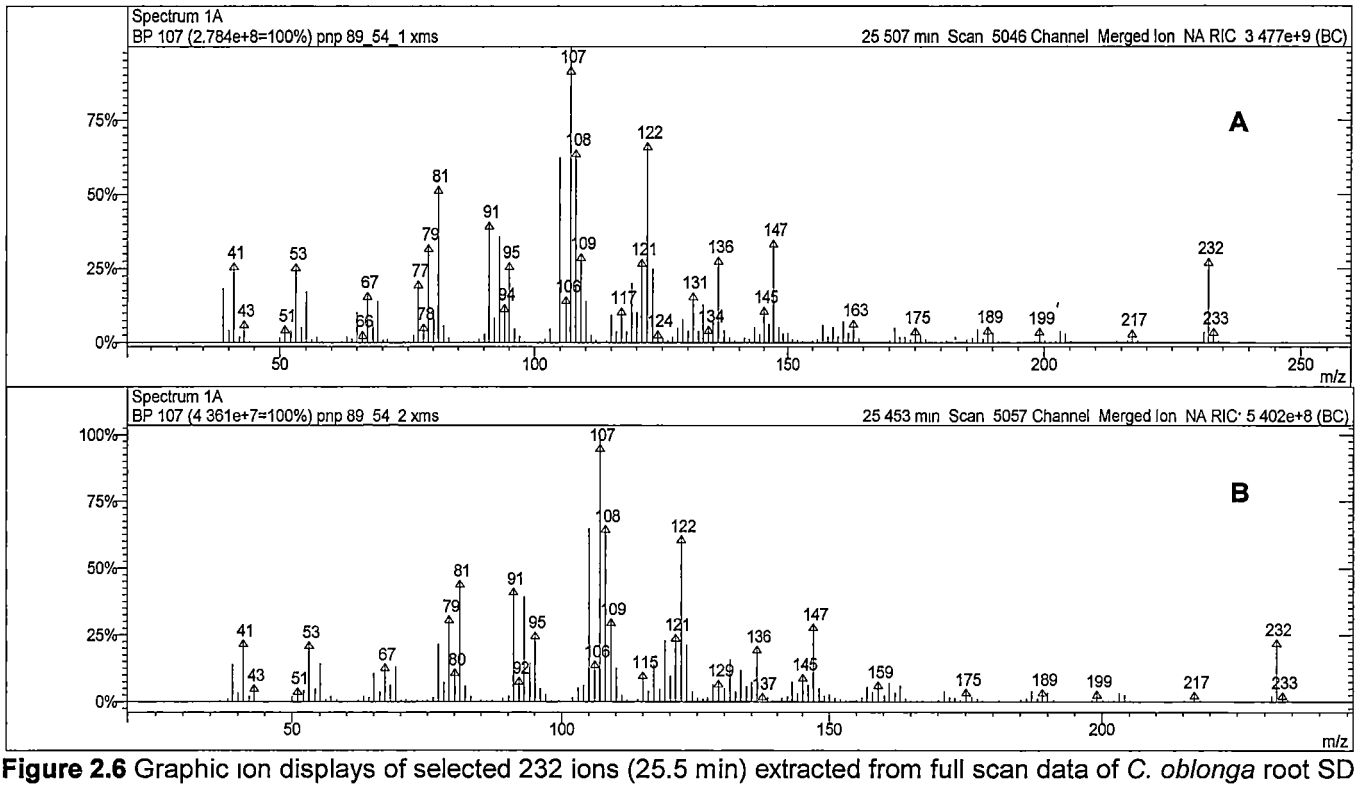


Figure 2.6 Graphic ion displays of selected 232 ions (25.5 min) extracted from full scan data of *C. oblonga* root SD oil (A) and *C. intratropica* (B) wood SD oil.

The extraction of *m/z* 232 ions from the full scan data of the *C. oblonga* oil showed that the sesquiterpene lactone that eluted at a retention time of 25.49 min had the same retention time (RT) as columellarin in the reference sample of *C. intratropica* wood oil. The MS spectrum as shown in the figure was identical to columellarin identified

by Doimo and colleagues (2001). Dihydrocolumellarin was identified in the same way. Columellarin altogether formed 30% of the total TIC of the oil.

Callitris spp. timbers are known for their resistance against termite attack. The durability of *Callitris* spp. timbers with respect to termite attack was reported to be due to the presence of columellarin (Watanabe *et al.*, 2005). Columellarin has been found to possess cytotoxic properties. It disrupted a variety of metabolic pathways and may result in degranulation of tissue mast cells with the liberation of histamine and other physiologically active compounds (Elissalde *et al.*, 1983). Columellarin has been reported only from *Callitris* spp. Its content in *C. columellaris* wood SD oil was 7%, *C. glaucophylla* SD wood oil contained 3%, and *C. intratropica* SD wood oil contained 3%. The percentage present in *C. oblonga* root oil was 30%, representing the highest columellarin content of any *Callitris* spp. oil. No other biological activities of columellarin have yet been reported. Table 2.4 compares the yields of columellarin obtained from other *Callitris* species.

Table 2.4 Comparison of the yields of columellarin obtained from different *Callitris* species

Species	Yield of SD oil (%)	Columellarin content in the oil	Yield of columellarin from the plant material (w/w %)
C. columellaris wood oil (Qld)	0 2	7%	0.014
C. glaucophylla wood oil (Qld)	0 3	3%	0 009
C. glaucophylla wood oil (NSW)	0.2	3%	0.006
C. intratropica wood oil	ND *	3%	ND *
C. oblonga root oil	0.2	30%	0 6

* Not determined

Sesquiterpene lactones have been studied for use in various ailments including their potential use as anti-inflammatory agents, (*Cichorium intybus* root extract), cancer (*Chloranthus henryi* root oil) and cardiovascular diseases (*Ixeris dentata* roots) (Ripoll *et al.*, 2007; Wu *et al.*, 2006; Baek *et al.*, 2004).

Guaiol, one of the major constituents in other *Callitris* spp., was present at very low concentration (<2%) in the root oils. Guaiol is often used in various personal care preparations (Baschong, 2005). Recently Yoshida and Mitsunaga (2006) patented a guaiol-containing preparation for its serum lipids lowering activity.

2.4.1.2 Leaf oils

The essential oils obtained from both *C. rhomboidea* and *C. oblonga* foliage exhibited a light green colour with a strong aroma. Steam distillation of *C. rhomboidea* leaves yielded a pale green oil (0.29% w/w). As shown in Table 2.2, 33 components were identified, accounting for 97% of the total essential oil. The oil predominantly contained α-pinene (42%), geranyl acetate (12%), neryl acetate (6%), and citronellyl acetate (7%) as the major monoterpenoid compounds. Unsurprisingly the oil also contained geraniol, nerol, and citronellol. The major sesquiterpene identified was thujopsene (2%). Unknown compound 7 (4%) which occurred not only in the aerial parts (leaves, bark and fruits of both plant species) but also in roots of both the plant species, remained unidentified.

Baker and Smith (1910), the pioneers who first studied *C. rhomboidea* plants reported the presence geranyl acetate in the foliage oil. A recent study by Brophy *et al.* (2007) reported on the chemical composition of oil from *C. rhomboidea* foliage collected from different locations within Australia. The main compounds reported from the leaf oils were geranyl, neryl and citronellyl acetates. The percentages and the major compounds in our oil were in agreement with the published data. Fig 2.7 shows the GC-MS traces of the SD foliage oil of *C. rhomboidea* and *C. oblonga.*

The essential oils obtained from the leaves of *C. oblonga* mainly contained monoterpenes (81%). The dominant monoterpenes were α-pinene (45%), isopulegol

(6%), citronellol (6%) and pulegol (2%). The major sesquiterpenes identified were columellarin (6%), spathulenol (2%), and cyclosativene (2%). Unknown compound 6 which formed 4% of the total oil remained unidentified. The SD oil of the *C. oblonga* foliage contained several sesquiterpene lactones including columellarin, dihydrocolumellarin, *cis*- and *trans*-methyl isocosticate, in contrast to none being reported from *C. oblonga* oils analysed by Brophy and colleagues (2007).

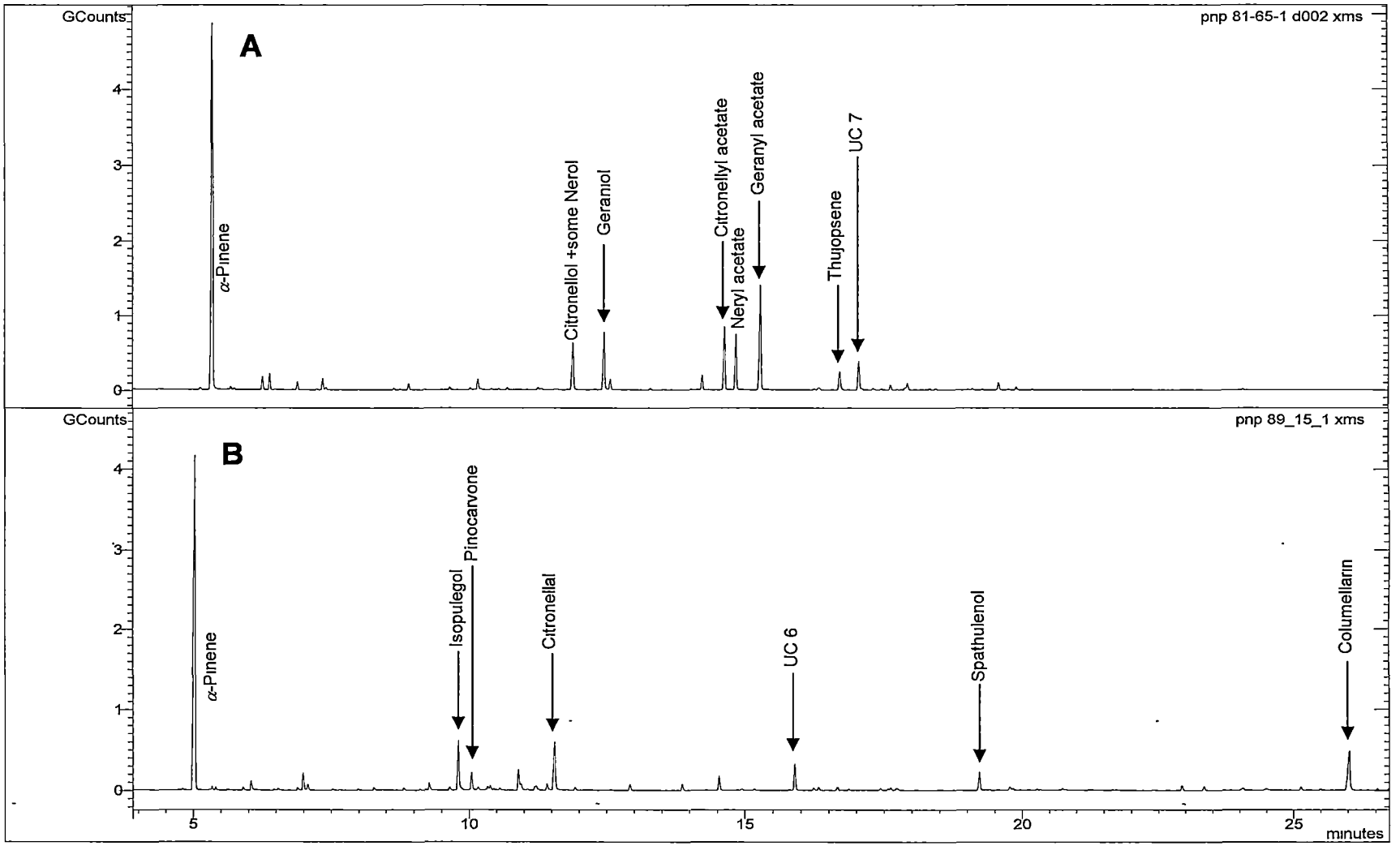


Figure 2.7 GC-MS chromatograms of SD foliage oils obtained from *C. rhomboidea* (A) and *C. oblonga* (B).

2.4.1.3 Bark and fruit oils

Hydrodistilled oils from the bark and fruit organs of *C. rhomboidea* were pale green in colour. In both the bark and fruit volatile oils, monoterpenes were the dominant components. A total of 28 compounds were identified from fruit oil, representing 98% of the total oils, while a total of 41 compounds were identified from bark oil representing 94% of the total oil. The major monoterpenoids detected in fruits were α-pinene (84%), myrcene (1%), and limonene (1%). In bark oil the major monoterpenes detected were α-pinene (57%), β-pinene (6%), δ-3-carene (5%), and myrcene (3%). The bark oil was characterised by the presence of many sesquiterpenes at low concentration levels. The major sesquiterpenes included thujopsene (1%) and guaiol (1%).

Monoterpenes constituted the dominant portion of *C. oblonga* SD fruit oil, occurring in a relative amount up to 89%, with α-pinene (74%) as the dominant compound. Isopulegol present in the leaf oils was completely absent in the fruit oil while limonene (7%) was found in a higher percentage than in the leaf volatile oil. The major sesquiterpenes were bicyclogermacrene (2%) and spathulenol (1%). Sesquiterpene lactones, which formed a major portion of components in the roots and leaf oil, were completely absent from the fruit oils. Fig 2.8 compares the GC-MS chromatograms of bark and fruit SD oil of *C. rhomboidea* while Fig 2.9 shows the GC-MS chromatogram of *C. oblonga* SD fruit oil.

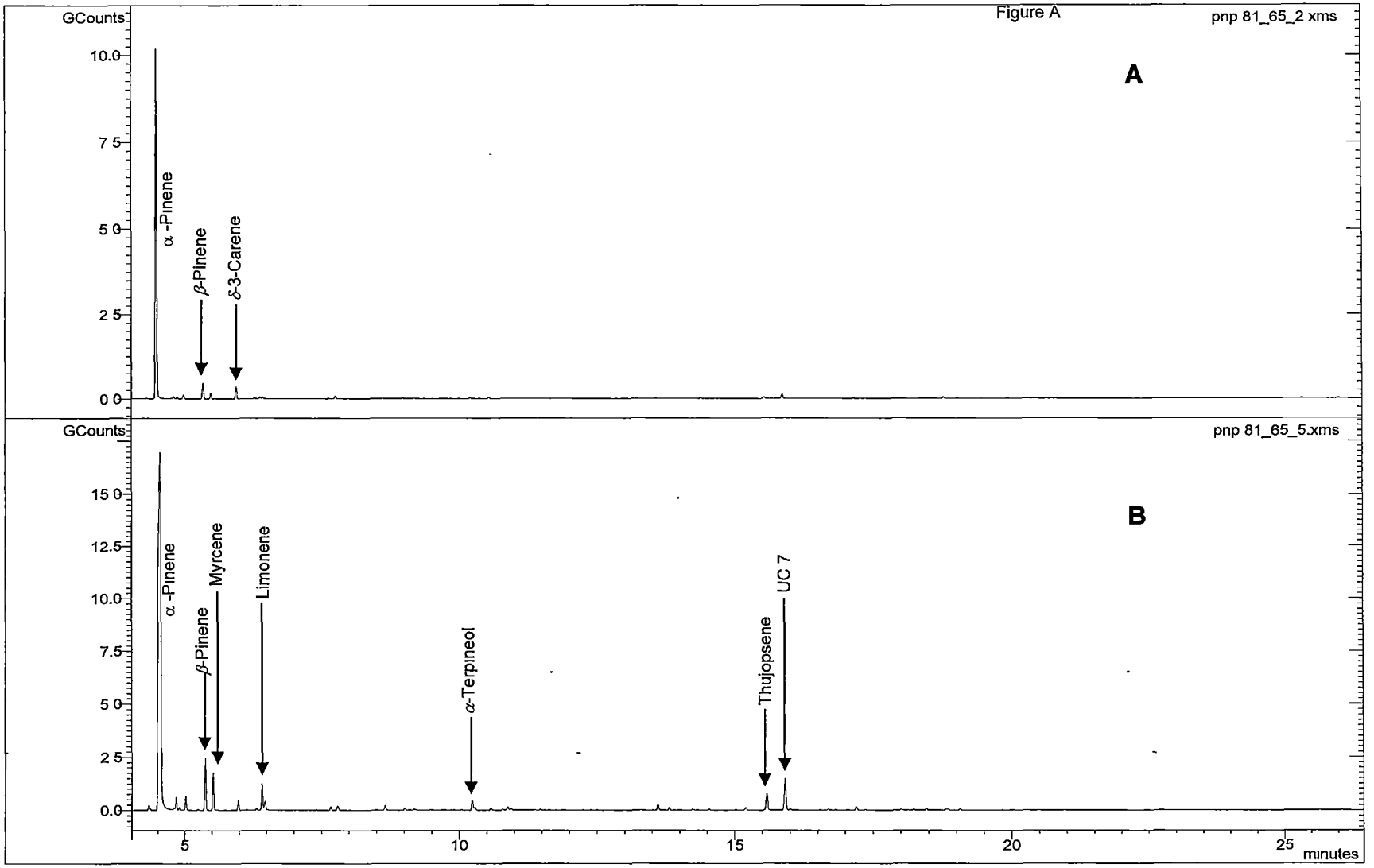


Figure 2.8 GC-MS traces of SD bark (A)and fruit (B) oils obtained from *C. rhomboidea*.

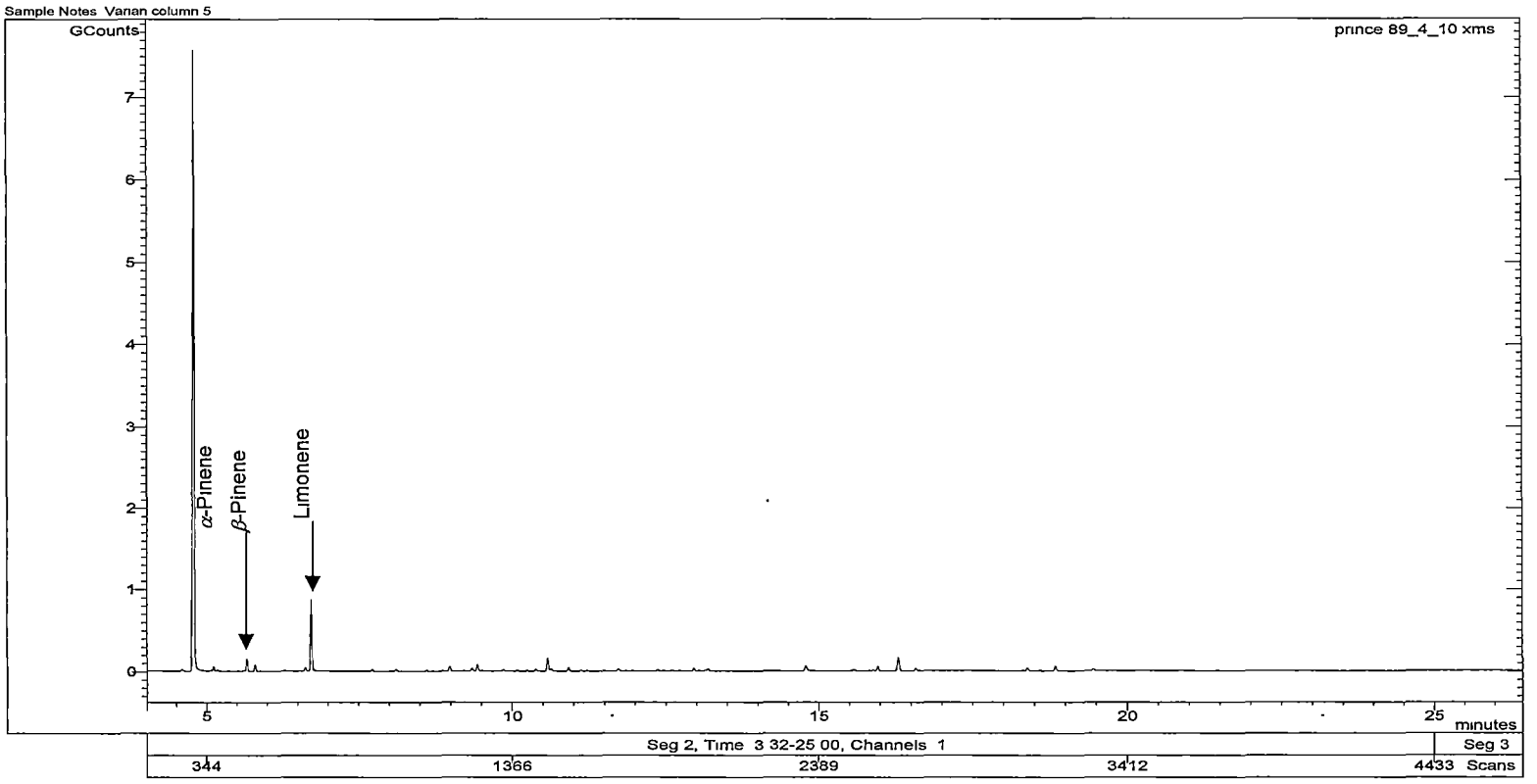


Figure 2.8 GC-MS traces of SD fruit oil obtained from *C. oblonga*.

2.4.2 Composition of the solvent extracts obtained from different organs of the *C. rhomboidea* and *C. oblonga* plants

The roots, leaves, stem, bark and fruits were extracted from both *Callitris* spp. Yields obtained from different organs of the plants are tabulated in Table 2.5.

Table 2.5 Yield of the extracts obtained from different organs of *C. rhomboidea* and *C. oblonga*

Plant parts	Mass of plant Material (g)	Extract weight (g)	% Yield (w/w)
C. rhomboidea root PE[a] fraction	260	0 25	0.1
C. rhomboidea root DCM[b] fraction	260	0 49	0.2
C. rhomboidea root MeOH[c] fraction	260	0.32	0.1
C. rhomboidea leaf PE fraction	780	6.40	0 9
C. rhomboidea leaf DCM fraction	780	7.52	1 0
C. rhomboidea leaf MeOH fraction	780	18.44	2.4
C. rhomboidea stem PE fraction	874	0 85	0.1
C. rhomboidea stem DCM fraction	874	0 91	0.1
C. rhomboidea stem MeOH fraction	874	5.47	0 6
C. rhomboidea bark PE fraction	820	2.70	0.2
C. rhomboidea bark DCM fraction	820	0.70	0 1
C. oblonga root PE fraction	320	0 59	0.2
C. oblonga leaf PE fraction	820	10 34	1.3
C. oblonga leaf DCM fraction	820	9.96	1.2
C. oblonga leaf MeOH fraction	820	4 64	0.6
C. oblonga stem PE fraction	964	0.93	0.1
C. oblonga stem DCM fraction	964	2.46	0.3
C. oblonga stem MeOH fraction	964	4 67	0 5
C. oblonga bark PE fraction	627	0 75	0 1
C. oblonga bark DCM fraction	927	0 63	0 1
C. oblonga bark MeOH fraction	627	1 02	0.2
C. oblonga fruit PE fraction	346	0.76	0.2
C. oblonga fruit DCM fraction	360	0 40	0.1
C. oblonga fruit MeOH fraction	380	0.39	0 1

[a]petroleum spirit [b]dichloromethane [c]methanol

In general the yield obtained from both the *Callitris* spp. was in the range of 0.1 to 2.5%. The highest yield (0.6-2.4%) was obtained from plant foliage, with the highest percentage from the polar MeOH fraction. The root and bark extracts from both *Callitris* spp. plants yielded between 0.1-0.2 % with no variation in the yields obtained from different organic solvents. The MeOH fraction obtained from the stem

of *Callitris* spp. yielded a higher quantity (0.5-0.6%) of material, while the fractions obtained by petroleum spirit (PE) and DCM extraction yielded between 0.1-0.2 %.

The dried extracts were reconstituted with a known volume of respective solvent (PE, DCM and MeOH) to make up a final concentration of 100 mg/ml. Non polar fractions (PE and DCM fractions) were analysed by GC-MS by using the same GC conditions as for the SD oil.

The fractions obtained from PE and DCM gave a similar profile of volatile components when examined by GC-MS. Hence, only volatile components identified from PE fractions are reported and tabulated in Table 2.5. Fractions obtained from all organs had significant amounts (>70%) of diterpenes. The only exception being the fractions obtained from roots of *C. rhomboidea* (37%) and *C. oblonga* (11%).

2.4.2.1 Root extracts

Out of the total 85.7% of the total TIC of the *C. rhomboidea* root sample, monoterpenes accounted for 11% while 38% and 36% were accounted for by sesquiterpenes and diterpenes respectively. The major monoterpenes identified were UC1 (7%) and neryl acetate (3%). As expected longiborneol (19%) detected also from SD oil constituted the highest percentage of sesquiterpene identified from the fraction. Other minor sesquiterpenes included longifolene (4%), UC7 (4%), δ-cadinene (4%) and UC13 (1%). Several unknown compounds such as UC5, 9, 11 and 12 previously analysed from SD oil, were absent in root solvent fractions. It was concluded that the major compounds analysed from root SD oil were also present in solvent extract fractions in similar relative proportions. The presence of diterpenes in solvent extracts fractions was an exception.

Table 2.6 shows the extract composition of *Callitris* spp. obtained from different parts of the plants as determined by GC-MS (percentage of the TIC). Few diterpenes were reported to be present in *Callitris* spp. Carman and colleagues (1970) revealed the presence of several diterpene acids such as sandaracopimaric acid, isopimaric acid, *cis*- and *trans*-communic acid from the oleoresin of *C. columellaris*. The GC data of these compounds were not available. Further down the years the same authors (1986) reported the presence of 3,10-dihydroxydiethylmentha-5,11-diene-4,9-dione from *C. columellaris* hexane neutral fraction with *m/z*(%): 332(53, M^+), 289(21), 271(11), 257(17), 243(19), 201(18), 191(11), 167(13), 166(30), 159(19), 150(11), 149(27), 137(16), 125(34), 124(17), 123(37), 43(100). None of the diterpenes found from our extracts matched the *m/z* data of the above-described compound.

Out of the total 95% compounds identified from the TIC of the *C. oblonga* root extract, monoterpenes constituted 3%, while late eluting γ-lactones represent the dominant fraction constituting 84%. Diterpenes accounted for 11% of the total TIC of the sample. The major monoterpenoid identified was UC1 (3%), while the dominant γ lactones identified were columellarin (45%) and UC23-30 together accounting for 21%.

Comparison with other *Callitris* spp. showed that the highest content of γ-lactones was in the root sample of *C. oblonga* (68%) as against *C. columellaris* (31%), *C. glaucophylla* (44%), and *C. intratropica* (61%) plant extracts. The key difference between the volatile composition of SD oil of the root and extract was the absence of several identified sesquiterpenes (>1%) such as longifolene, β-elemene, thujopsene; while noticeably several sesquiterpenes such as UC5, 7, 9, 10, 12, and 17-19 were also absent in the solvent extracts. Most importantly the percentage of γ-lactones from

Table 2.6 Petroleum ether extract composition of *Callitris* spp obtained from different parts of the plant as determined by GC-MS (TIC). Peaks are listed in elution order from the VF-5ms column

Compound	KI	*C. rhomboidea* leaf	*C. rhomboidea* fruit	*C. rhomboidea* bark	*C. rhomboidea* root	*C. oblonga* leaf	*C. oblonga* fruit	*C. oblonga* bark	*C. oblonga* root	*C. oblonga* stem
α-Pinene	935	4 1	11 6	14 4		16.4	10 2	12 8		4 7
Camphene	953				0 5					
β-Pinene	974	0 6		1 0			0.3	0 3		0.2
Myrcene	988		0.3	0 4		0 4	0.1	0 2		
δ-3-Carene	1009	0.8		1 4			1 1	0.5		
β-Phellandrene	1030			0 3						
Limonene	1032	0.2		0 2		0 3	0 4	0 4		
Citronellol	1150					4.3				
Unknown compound 1	1223				7 4				3 0	
Geraniol	1253	0 8								
Citronellyl acetate	1349	1 6								
Neryl acetate	1358	1.9			2.8					
Geranyl acetate	1378	2.9								
Longifolene	1419				4.1					
Carveol	1423					0.4				
Thujopsene	1444	1.4			2.3	0.5	0 4	0.1		
Unknown compound 7	1461	3 1			4 3	1.1	1.1	0 4		
δ-Cadinene	1525				4 0					
Spathulenol	1588	1 1								
Guaiol	1604				1 3					
Longiborneol	1616				19 7					
Unknown compound 13	1622				1 4			0.2		
Unknown compound 14	1649							3 4		
Unknown compound 15	1686							5 9		
Dihydrocolumellarin	1929							1.9		
Columellarin	1984							44 9		
Unknown compound 23	1988							1 0		
Unknown compound 24	1992							1.5		
Unknown compound 25	1994							3 9		
Unknown compound 26	2004							7.7		
Unknown compound 27	2010							2.1		
Unknown compound 28	2015							1.1		
Unknown compound 29	2021							3.3		
Unknown compound 30	2020							0.9		
Diterpenes		70.9	78.0	79.7	36.9	83 9	73 5	87.0	10.9	92.5
Total		91.9	90.7	98 3	84 7	98 6	96.9	87.0	94.6	97.4

the solvent extract totalled to 67% while in SD oil it added up to 46% of the total TIC of the samples. The yield of the samples remained the same. Solvent extracted samples could be used as better source of columellarin, which already has been studied for its activity against termites and for other biological activities.

2.4.2.2 Leaf extracts

Contrary to our expectation out of the total 92% of the TIC of the *C. rhomboidea* foliage PE fraction monoterpenes represented only 15% where as 88% of the total SD oil were monoterpenes. Sesquiterpenes constituted 6% and diterpenes amounted to 70.9% of the total oil. The chief monoterpenoids compounds were α-pinene (4.1%), citronellyl acetate (2%), neryl acetate (2%) and geranyl acetate (9%) whilst sesquiterpenes identified were UC7 (3%), thujopsene (1%) and spathulenol (1%). As expected the alcohols of the respective acetates were also present in the sample at below 2%. Out of the total 99% of TIC of the sample, monoterpenoids accounted for about 21.4% whilst sesquiterpenes constituted 2%. As in the former leaf sample, diterpenes accounted for about 84%. The major monoterpenoids identified were α-pinene (16%) and citronellol (4%). The absence of some monoterpenoids such as pulegol and isopulegol and the sesquiterpene lactone columellarin were the major differences between the SD oil and the solvent extract.

2.4.2.3 Bark and fruit extracts

As shown in Table 2.6, out of the total 98% of the TIC of *C. rhomboidea* solvent bark extracts, and 91% of the *C. rhomboidea* fruit solvent extract monoterpenes accounted for 18% in bark extract and 12% in fruit extract. The major compound was *α*-pinene in both the extracts. The chief components in both the solvent extracts were diterpenes accounting for 80% of the total extract in bark and 71% in the fruit. A noticeable

difference between SD oil and solvent extracts were the absence of sesquiterpenes in the latter.

2.4.2.4 Wood extract

GC-MS analysis of *C. rhomboidea* stem solvent extract provided no evidence of the presence of monoterpenes or sesquiterpenes. GC-MS analyses of *C. oblonga* stem extract indicated the presence of monoterpenes such as α-pinene and β-pinene, but sesquiterpenes were lacking in the sample. Absence of sesquiterpene lactones such as columellarin and dihyrocolumellarin in the solvent extracts was the most noticeable difference. Earlier studies done on wood extracts of *Callitris* spp. (*C. columellaris*, *C. glaucophylla*, and *C. intratropica)* illustrated the similarity of the volatile composition of their respective wood oil. Their respective SD wood oil and solvent extracts were rich in sesquiterpene lactones. GC-MS chromatograms of all the extracts are shown in Appendix 1.

2.5 CONCLUSION

Constituents of the root, leaf, bark and fruit oils of *C. rhomboidea* and *C. oblonga* have been established by steam distillation and solvent extraction. Root oils were significantly rich in sesquiterpene lactones such as columellarin, where as leaf, fruit and bark oils were rich in monoterpenes. Volatile components present in each plant parts, varied between the two different *Callitris* species.

CHAPTER 3

The Release of Volatile Compounds from *Callitris* Spp. under Unstressed (In Situ) and Stressed (In Vial) Experimental Conditions, Determined by SPME.

3.1 INTRODUCTION

Solid phase microextraction (SPME) is a solvent free sampling technique. It was developed by Pawliszyn and co-workers (1989) to overcome the ineffectiveness of solid phase extraction (SPE) and liquid-liquid extraction (LLE) in some applications. It was developed as a preconcentration technique to analyse pollutants in water. SPME is a very popular tool used in a variety of fields (Fig 3.1), and has increasingly been applied in environmental studies of pesticides, herbicides, volatile organic compounds in landfill soils, organometallic compounds in sediments of soil; in food chemistry to determine the quality of fresh and processed foods and food adulteration; in biological fields for urine analysis for a variety of drugs like 3,4–methylenedioxymetamphetamine (MDMA, ecstasy), antidepressants, corticosteroids; and several other applications (Kataoka, 2002; Bosset *et al.* 2002).

SPME is an efficient technique which is a fast and simple method of collecting volatiles at detection limits less than parts per billion by volume. In this approach coated fused silica fibres are equilibrated with the sample that needs to be analysed. Analytes that are directly adsorbed on to the coated fused-silica fibres are then thermally desorbed into a gas chromatograph (GC) injection port. Different SPME fibres and coatings such as 100 μm polydimethylsiloxane (PDMS) or divinyl benzene

(DVB), can be used for specific applications. For example 100 μm PDMS fibres were used for the analysis of volatiles from plants, whereas 7 μm PDMS fibres were used for the analysis of nonpolar high molecular weight compounds (Tholl *et al.*, 2005).

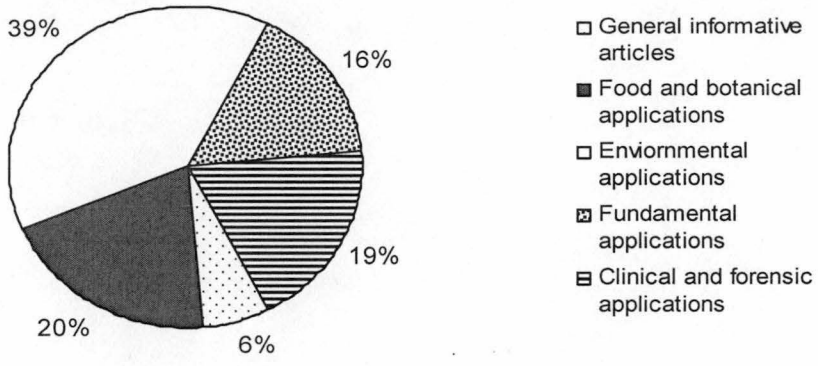


Figure 3.1 Distribution of publications dealing with SPME (Pillonel *et al.*, 2002).

The method is useful as an inexpensive tool for the identification of volatiles from herb extracts like eucalyptus, rosemary and thyme oils. It could avoid the introduction of concentration errors, environment pollution, health hazards to the personnel working and extra cost of the solvents. A disadvantage of commonly-used steam distillation is it is often destructive in nature, giving rise to artefact formation by the decomposition of various compounds at elevated temperature in the presence of water. Solvent extraction has the disadvantage of consuming large amounts of solvent and thereby leading to potential loss of metabolites or plant material (Sarkar *et al.*, 2006).

The primary disadvantage of the SPME technique is that it will not allow the trapping of sufficient amounts of volatiles for structure elucidation. All the emitted plant

volatiles are not removed through one sampling time, making changes in emission over time difficult to determine. SPME is a tool for qualitative analysis rather than quantitative measurements of changing volatile emissions over time (Tholl *et al.*, 2005).

Plants not only liberate non-organic volatiles (carbon dioxide and oxygen) during photosynthesis, but some plants also liberate volatile organic compounds to the environment from different parts of the plant, like leaves, flowers and fruits. According to Tholl and colleagues (2005), non-destructive headspace analysis forms the most effective tool for detecting the volatiles released into the environment compared with the traditional methods of solvent and steam distillation. An overall choice of which system to use in a particular experiment for collection and analysis of plant volatiles usually depends on the biological problem and plant material being investigated (Fig 3.2).

The aromatic characteristics of essential oils provide various functions for the plants including attracting or repelling insects; protecting themselves from heat or cold; and utilizing the constituents in the oil as anti-bacterial or anti-fungal agents. Released volatiles have been extensively studied for above-ground plant parts but not many studies (Steeghs *et al.*, 2004) have yet been done on underground parts (root emissions).

The release of volatiles from the roots of cypress plants has not been a subject of study to date. The roots of potted *Callitris rhomboidea* and *Callitris oblonga* plants when uprooted from their pots produced a strong aroma due to the evolution of volatiles. *C. rhomboidea* and *C. oblonga* contain reasonably high levels of volatile organic compounds in their roots. These oils probably have a biological role such as

protection against fungal or bacterial organisms or as feeding deterrents. Release of oils from the roots into the soil and surrounding environment may occur in order to facilitate a biological effect.

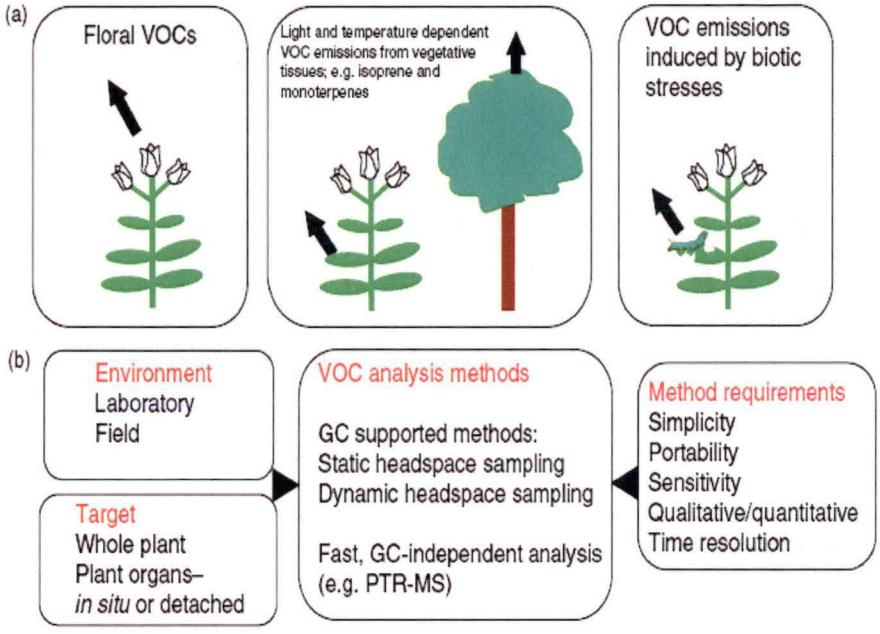


Figure 3.2. Strategies for plant volatile analysis (adapted from Tholl *et al.*, 2005)
(a) Typical sources of plant volatile organic compounds (VOC) emissions.
(b) Considerations for planning VOC analysis experiments

This work describes the application of SPME to the characterisation and quantification of volatile compounds emitted from the living roots (*in situ*) of *C. rhomboidea* plants. The plant root samples and leaves were also analysed by 'in vial' experiments to analyse the release of volatiles upon damage to the root structure. The objective of the study was to determine whether terpenes are released from the roots of *Callitris* spp. using SPME sampling *in situ*.

3.2 EXPERIMENTAL

3.2.1 Materials

Potted young healthy plants of *C. rhomboidea* and *C. oblonga*, approximately 2 years old in 25 cm pots, bought from Pulchella Nursery, Buckland, were used for 'in pot' experiments.

GC-MS vials with septum and a screw cap of approximately 2.5 cm (length) and 0.5 cm (diameter) were used for keeping the plant parts (foliage and roots) for equilibration and SPME sampling. Beach sand washed with 10% sulphuric acid LR (> 98%) (BDH, Melbourne, Australia) was used for the model experiment. Geranyl acetate (98%) and cineole (98%) were bought from Sigma Aldrich (Sydney, Australia).

3.2.2 Instruments

3.2.2.1 SPME fibre with holder

Fused silica fibres (75μm) coated with PDMS, and Supelco manual SPME syringe were obtained from Supelco (Sydney, Australia). The schematic of the fibre, needle and plunger is given in Fig 3.3.

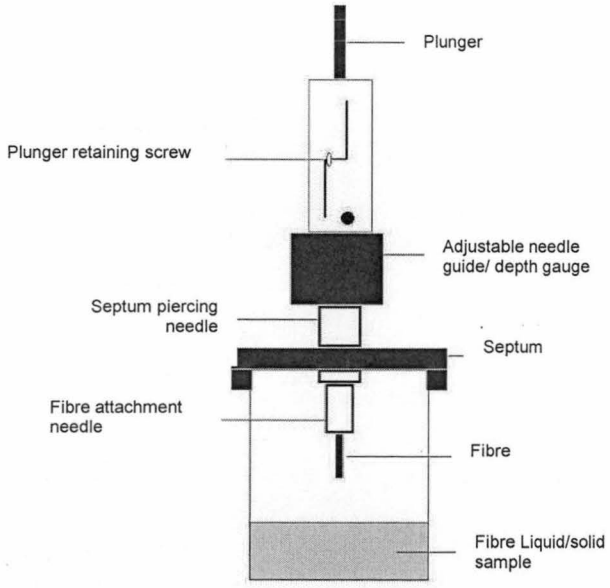


Figure 3.3 Solid Phase microextraction fibre with the plunger (Adapted from The University of South Carolina, 2007)

A probe to penetrate the soil and to enable SPME sampling of volatiles released from the soil was designed and made 'in house'. This consisted of a glass syringe body approximately 10 cm long joined to a threaded section obtained from a wisp vial. This enabled a screw cap with septum to be attached to one end of the syringe body. A 15 cm, 17 gauge needle was perforated transversely, using a fine cutting wheel, at intervals of approximately 1 cm down its length. The end of the needle was cut off squarely and stopped with a teflon plug having a blunt point and a diameter in excess of the needle diameter. The needle was joined to the modified syringe body using teflon tape over the Luer fitting to ensure a gas tight seal (Fig 3.4).

Figure 3.4 Soil probe with needle.

3.2.1.2 Gas Chromatography-Mass Spectrometry (GC-MS)

SPME fibres were desorbed and analysed using a Varian 3800 GC coupled to a Varian 1200 triple quadrupole mass spectrometer (MS). The GC column was a Varian VF-5 ms (Factor four from Varian) (30m x 0.25mm internal diameter and 0.25 micron film), using Varian 1177 split/splitless mode. Injector temperature was 280 °C, with a desorption time of 3 min. GC oven temperature (cryo option used) was 10 °C for 2 min then to 33 °C at 23 °C per min, and then to 280 °C at 8 °C per min. Carrier gas

(helium) flow rate was 1.2 ml per min in constant flow mode. Split valve was turned on at 2 min. The range from *m/z* 35 to 350 was scanned every 0.3 s. Typical multiplier setting was 1300 volts and data was processed with Varian Star software. Ion source was 220 °C, and the transfer line was held at 290 °C. All the GC-MS hardware and software was purchased from Varian Inc, Melbourne, Australia.

Steam Distilled (SD) oil was analysed by GC-MS on the same instrument with slight modification. Oven temperature profile was 60 °C for one min then to 210 °C at 6 °C per min then programmed to 270 °C at 25 °C per min with a 5 min hold at 270 °C. Samples were injected split (20:1). The ion source temperature was 220 °C and the transfer line was held at 290 °C. All other conditions were as for SPME analyses.

3.2.2 Methods

The study methods were divided into 'in vial' study methods, 'model method' based on sand and *in situ* experiments. *In situ* experiments were further divided in to 'in ground' experiments and 'in pot' experiments. All the samples were collected by using the SPME sampling technique and the results were analysed by GC-MS.

3.2.2.1 'In vial' experimental model

'In vial' experiments were performed on the collected roots and leaves of nursery grown plants of *C. rhomboidea* and *C. oblonga*. Around 0.5 g of roots or 10 leaves of *C. rhomboidea* and *C. oblonga* were placed in different GC vials. The vial was screw capped and allowed an equilibration time of 15 mins at room temperature. The SPME fibre was introduced into the vial containing the sample through the septum and the fibre was exposed to the air above the plant material inside the vial for 20 mins. The

fibre was desorbed into the GC port immediately after its removal from the sample vial. Desorption time was 3 mins for all runs. Each sample was analysed by GC-MS.

3.2.2.2 Steam distillation

The roots of the same *C. rhomboidea* and *C. oblonga* plants previously used for 'in vial' and 'in pot' experiments were hydrodistilled in a Clevenger-type apparatus as described in Chapter 2. Oil was collected and stored at a temperature of 4 °C, until used in the experiments.

3.2.2.3 Model system

A 'model system' based on sand was designed to analyse the efficiency of the probe and the time required for the soil volatiles to equilibrate with the soil probe for SPME analysis. A known quantity of terpenes commonly occurring in *Callitris* spp. was mixed with a known mass of sand particles. This standard sand/terpene mixture was then serially diluted with fresh sand to obtain eight dilutions. The sampling procedure used the same protocol as the *in situ* sampling technique.

Steam distilled root oil, 1,8-cineole, and geranyl acetate; (100 mg of each) were dissolved in 100 ml of petroleum spirits in a round bottom flask (500 ml). Acid washed sand (100 g) previously dried in an oven at 100 °C for 24 hrs was added into the round bottom flask to form a stock mixture. The contents in the flask were rotary evaporated at reduced pressure at a temperature of 25 °C for 45 mins to remove petroleum spirit. Rotary evaporation possibly facilitated a uniform coating of essential oil constituents over the sand particles.

A sample of the essential oil solution was collected before rotary evaporation and analysed by GC-MS. A sample of the stock sand mixture (90 g) was mixed with 90

ml of petroleum spirits. The resulting essential oil solution was collected and analysed by GC-MS to determine any loss of volatile constituents during rotary evaporation of the sand/essential oil mixture.

The stock sand/essential oil mixture was then serially diluted by mixing 10 g of stock sand/essential oil mixture with 90 g of fresh sand in a 250 ml conical flask to obtain eight dilutions. Each dilution of sand sample in a conical flask was hand tumbled for 20 mins before the next serial dilution was made. This ensured a uniform distribution of essential oil coated sand particles with the fresh sand. Each flask containing sand sample was covered with aluminium foil to minimise the loss by volatilisation of essential oils constituents.

Sand samples (60 g) were put into 50 ml test tubes having a diameter of 3 cm. Prior to using, the soil probe was cleaned with water and detergent and oven dried (100 °C) for 24 hrs to remove volatiles. The soil probe was inserted into a sand-filled test tube to a depth of 6 cm. The soil probe (Fig 3.4) was allowed to equilibrate with the sand volatiles for 40 mins. The SPME fibre was then introduced into the soil probe through the septum and allowed to equilibrate with the volatiles for 40 minutes.

SPME sampling was carried out starting with the least concentrated sample. The least concentrated sample was dilution number 8 and the most concentrated sample being dilution number 1. After sampling, the SPME fibre was desorbed in the injection port of the GC-MS instrument. Further sand/essential oil sample dilutions were analysed by SPME using the same protocol.

In order to determine whether longer exposure time would increase the detectability of volatiles, dilutions 1 and 3 were sampled by exposing the SPME fibres for an

extended period of 16 hours. The volatiles adsorbed on the SPME fibres were then analysed by GC-MS.

3.2.2.4 In situ experimental model

3.2.2.4.1 Collection sites

For 'in ground' experiments, samples were separately collected from two different locations in Tasmania. The first sample collection site on the University of Tasmania campus near the University Herbarium and the latter sample site was a private garden at 23 Scott Street, Bellerive, Hobart. The trees were 7-10 years old.

3.2.2.4.2 'In ground' experiments

At the University of Tasmania campus site the soil near the collection site was covered with pine bark mulch plant litter and twigs of the *C. rhomboidea* plants. These were removed and the soil probe was plunged into the soil to 6 cm depth, about 15 cm from the trunk of the tree. The soil probe was allowed to equilibrate with the soil volatiles for 40 minutes. The SPME fibre was then introduced into the soil probe through the septum and allowed to absorb the volatiles for 40 minutes. The needle was then removed, followed by desorption to the injection port of the GC-MS systems. A second sampling was performed at 30 cm from the trunk of the same plant using an identical protocol.

At the Bellerive site a light covering of mulch was removed from the sampling site, which was approximately 1 m from the trunk of the tree in a zone of high root mat density. An identical sampling protocol was used for the sampling as used at the University of Tasmania campus site.

3.2.2.4.3 'In pot' experiments

'In pot' experiments were performed on potted nursery-grown *C. rhomboidea* and *C. oblonga* plants. The collection method followed was the same as that for the 'in ground' experiments except that the probe insertion was done at a distance of 10 cm from the plant stem.

3.2.3 Identification of Terpenoids by GC-MS

Identification of the terpenoids was performed by the same procedure as is described in Chapter 2.

3.3 RESULTS AND DISCUSSION

3.3.1 'In vial' experiment

The 'in vial' experiments were performed to demonstrate the suitability of SPME for the analysis of root volatiles and to demonstrate which volatiles could be detected. GC-MS analysis of 'in vial' samples of roots exhibited a large number of volatile compounds characteristic of the *Callitris* spp. SD roots oils. The major volatile constituents in the *C. rhomboidea* SD root oil were longifolene, thujopsene, longiborneol, guaiol, and UC1 with MW 180. The major compounds (>5%) detected by GC-MS from 'in vial' SPME samples were longifolene, δ-cadinene, camphene, tricyclene, UC1, and longiborneol. Figs 3.5 and 3.6 demonstrate the comparison of the GC-MS traces of the SD root oil and 'in vial' SPME sampling of the roots.

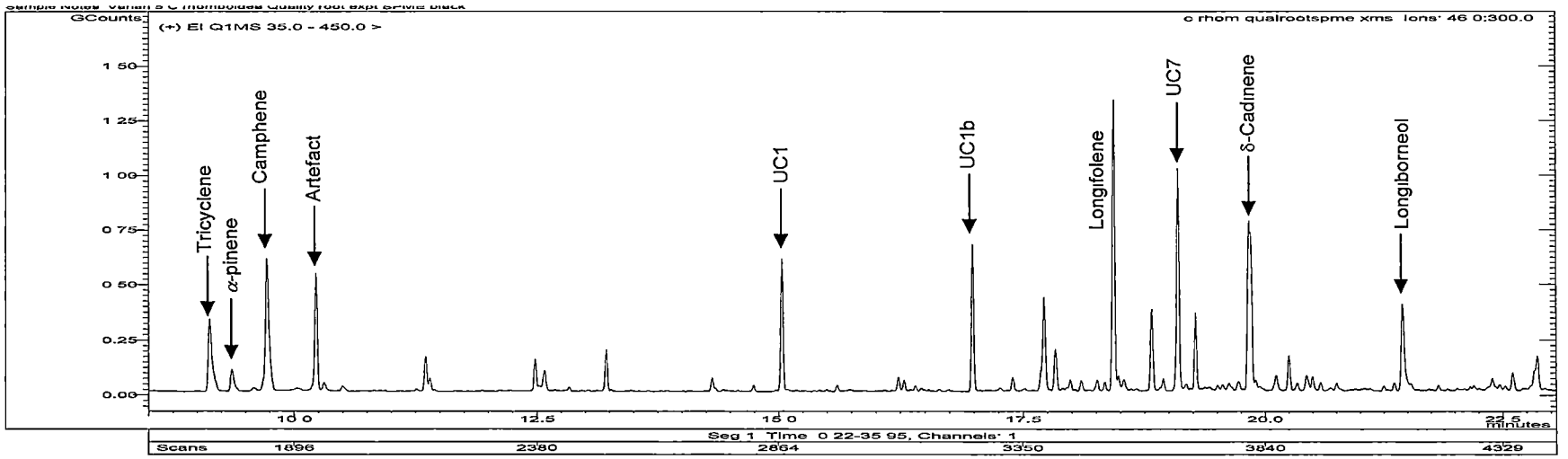


Figure 3.5 GC-MS chromatograms of the 'in vial' SPME samples of *C. rhomboidea* root.

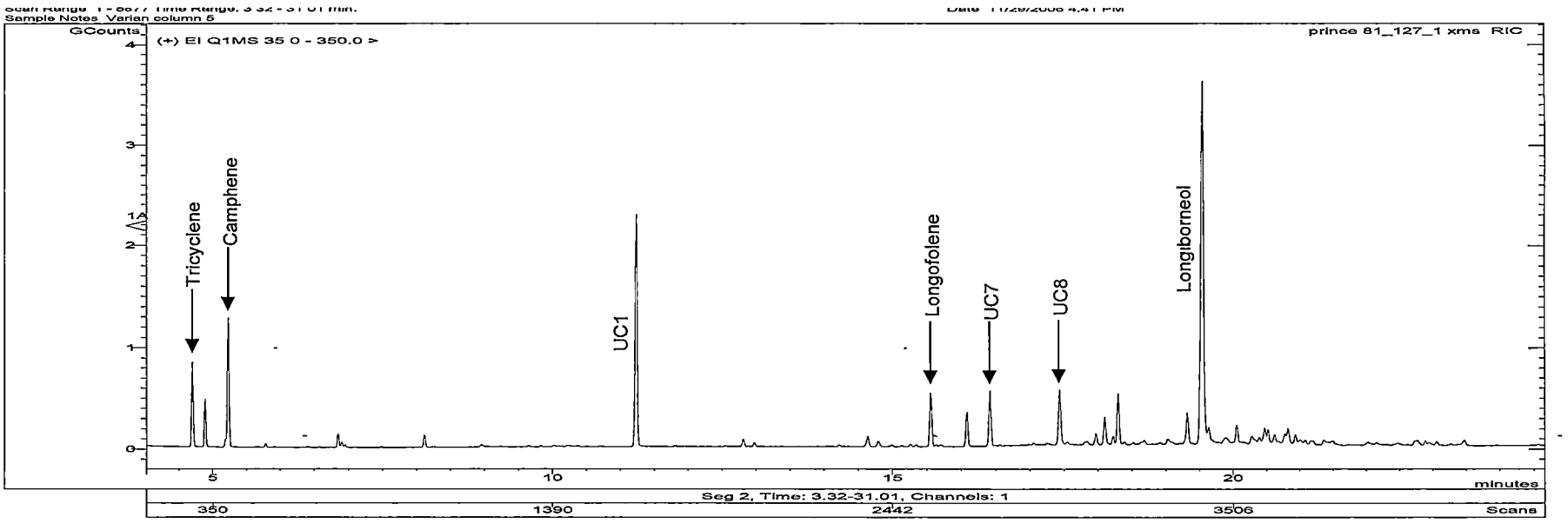


Figure 3.6 GC-MS chromatograms of the SD oil of *C. rhomboidea* roots

As was described in Chapter 2, UC1 was characteristic of the SD root oils of *Callitris* plants. Accurate mass measurement of UC1 was undertaken and was found to be 180.11456 amu corresponding to $C_{11}H_{16}O_2$.

The most surprising result was the presence of four unidentified compounds UC34 (1%), UC35 (6%), UC36 (1%), and UC37 (3%) with a mass of 180 amu in the 'in vial' experiments along with UC1. These compounds could possibly be isomers of UC1. Comparisons of the mass spectral data are shown in Fig 3.7. The mass spectral data of the unknown compounds were *m/z* (%): UC34 180(45), 165(9), 148(42), 139(14), 125(25), 121(100), 120(28), 119(30), 112(24), 105(66), 97(12), 88(56), 80(10), 79(36), 77(39), 65(13), 44(54), 44(54), 43(21); UC35 180(54), 165(10), 152(10), 149(15), 148(96), 137(9), 133(21), 121(47), 120(34), 119(30), 106(15), 105(46), 94(36), 93(36), 92(48), 91(41), 81(18), 79(47), 77(31), 68(100), 67(71), 59(12), 55(12), 53(32); UC36 180(45), 165(14), 149(9), 121(100), 119(15), 105(57), 93(15), 91(25), 79(20), 77(20), 44(20) and UC37 180(34), 165(12), 149(9), 121(100), 105(47), 93(11), 91(22), 79(16), 53(5), 43(6). Figs 3.8 and 3.9 demonstrate the comparison of the evolution of the unknown compounds (UC34-UC37) with MW 180 from the SD oils and the 'in vial' experiments.

The chromatogram of the SD oil was scanned for *m/z* 180 ions, but none of the unknown compounds UC34-UC37 were found in the SD oil. On analysing the mass spectral data of the unidentified compounds, UC34, UC36 and UC37 had a base peak of mass 121amu while UC35 had a base peak of 68 amu.

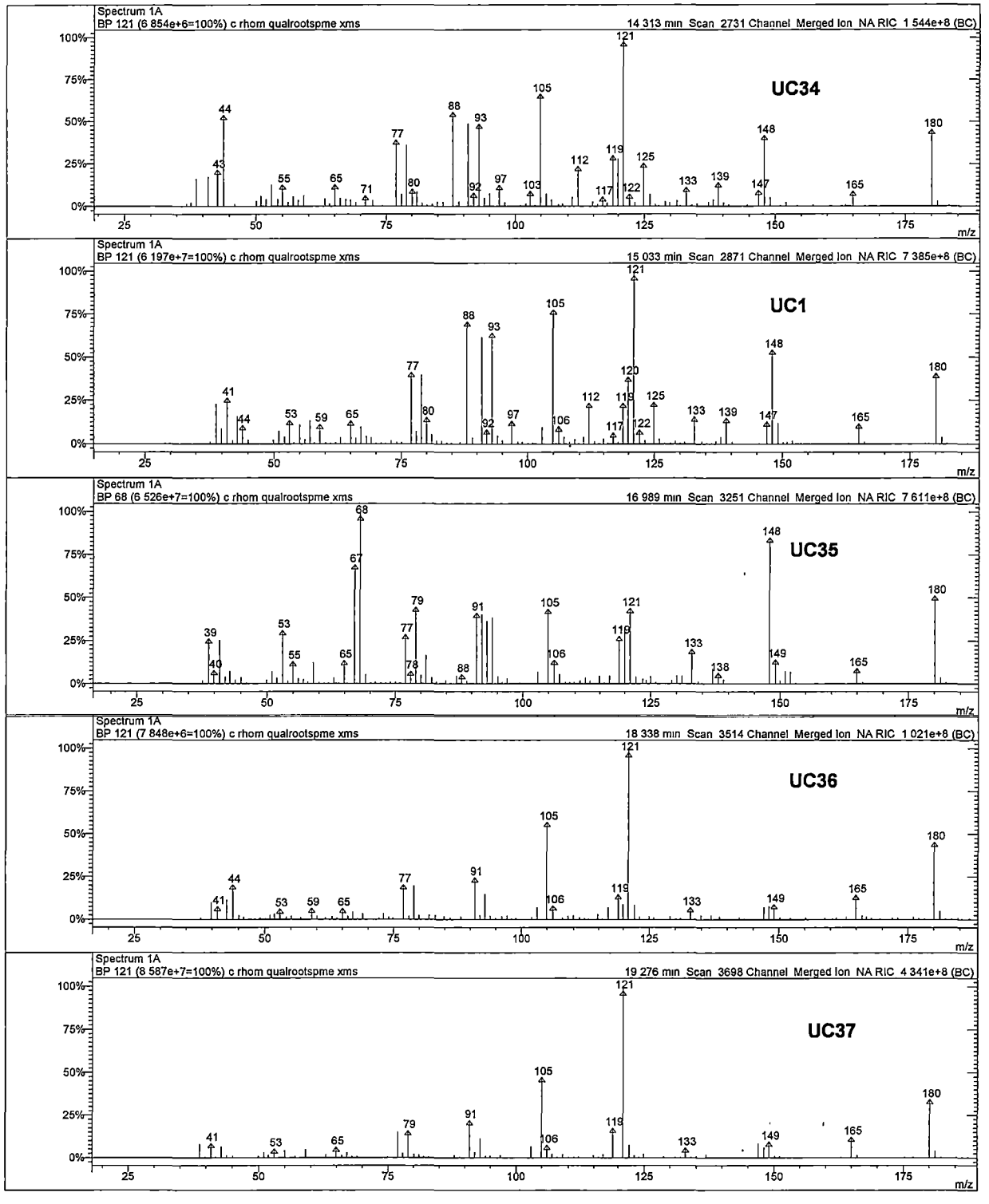


Figure 3.7 Mass spectra of the unknown compounds (UC1, UC34-UC37) found by SPME 'in vial' sampling of *C. rhomboidea* roots

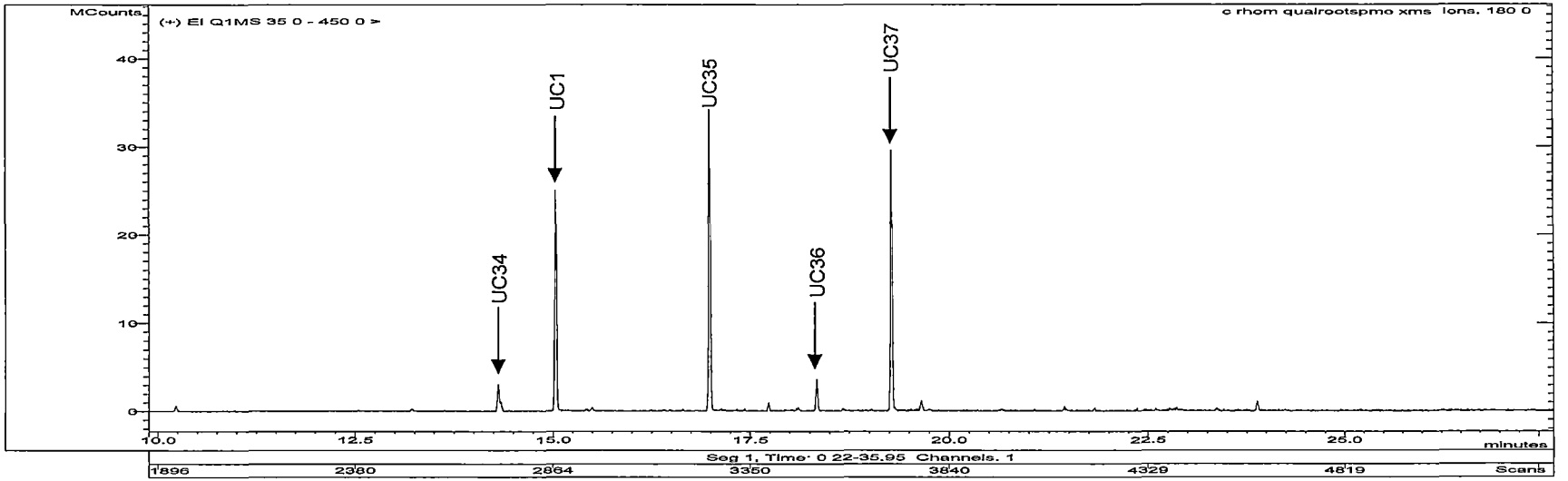


Figure 3.8 Evolution of the UC's (34-37) with MW 180 from the 'in vial' root experiments of *C. rhomboidea* plants The chromatograms are of the intensity of the ion at *m/z* 180

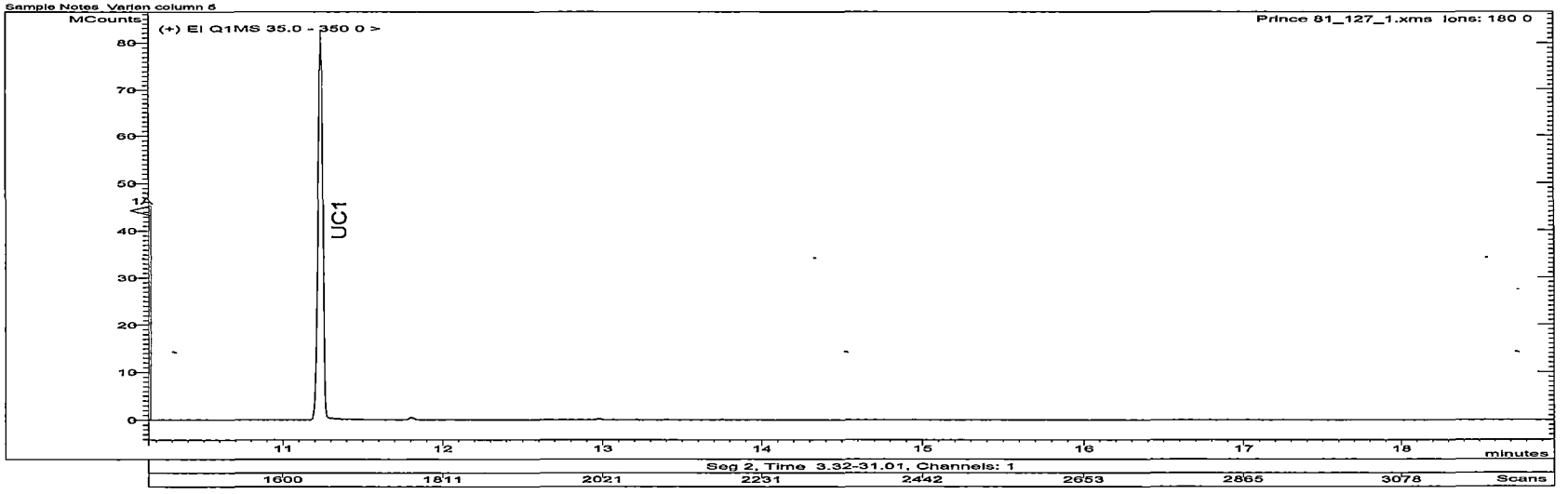


Figure 3.9 Absence of unknown compounds (UC34-UC37) from the SD root oil of *C. rhomboidea* plants. The chromatograms are of the intensity of the ion at *m/z* 180.

It was assumed that since the unknown compounds (UC34-UC37) were ester compounds, these compounds could have hydrolysed during SD (Simandi *et al.*, 1996) or the compounds could have been destroyed by heat during SD. Supercritical fluid extract (SFE) of *C. rhomboidea* roots obtained during my previous research study (Philip, 2007), and analysed for the presence of unknown compounds (UC34-UC37), but the compounds were not detectable in the SFE extract (Old SFE data was reinterpreted). The composition of the SFE extract was similar to that of the SD extract, with similar proportion of major constituents. It could thereby be concluded that the unknown compounds (UC34-UC37) with MW 180 amu could only be detected by the SPME method.

Analysis of the *C. oblonga* 'in vial' root samples using the same protocol, exhibited a release pattern of volatile compounds characteristic of the respective SD root oil (Figs 3.10 and 3.11). The major monoterpenoids detected from the 'in vial' sample were camphene (6%), tricyclene (4%), UC1 (4%), and α-pinene (2%). Sesquiterpenoids identified were columellarin (30%), β-elemene (7%), and UC8 (2%). The most characteristic compound was columellarin, a sesquiterpene lactone having a molecular weight of 232, which constituted about 30% of the TIC of SD root oil of *C. oblonga* plants, and was also detected in the SPME sample (30% TIC of the SPME sample). Unknown compounds (UC34-UC37) assumed to be isomers of UC1, were also present in *C. oblonga* root oil analysed by SPME.

The SPME method was also employed to study the elution of volatiles from 'in vial' samples of the leaves of *Callitris* plants. *C. rhomboidea* SD leaf oil predominantly contained α-pinene (42%), geranyl acetate (12%), neryl acetate (6%), and citronellyl acetate (7%) as the major monoterpenoid compounds. But these three acetates which

were at relatively high levels in the SD oil were not detected in the SPME 'in vial' sample analysis of the leaf. The main compounds that could be detected by GC-MS analysis were β-pinene and limonene (Fig 3.12 and 3.13).

The dominant monoterpenes in the SD oil of *C. oblonga* were α-pinene (6%), isopulegol (6%), citronellol (6%) and pulegol (2%). The major sesquiterpenes identified were columellarin (22%), spathulenol (2%), and cyclosativene (2%). Other than α-pinene, none of the said compounds could be detected by SPME analysis of the 'in vial' foliage sample. The chromatograms of the SD leaf oil and SPME 'in vial' samples of *C. oblonga* are presented in Figs 3.14 and 3.15.

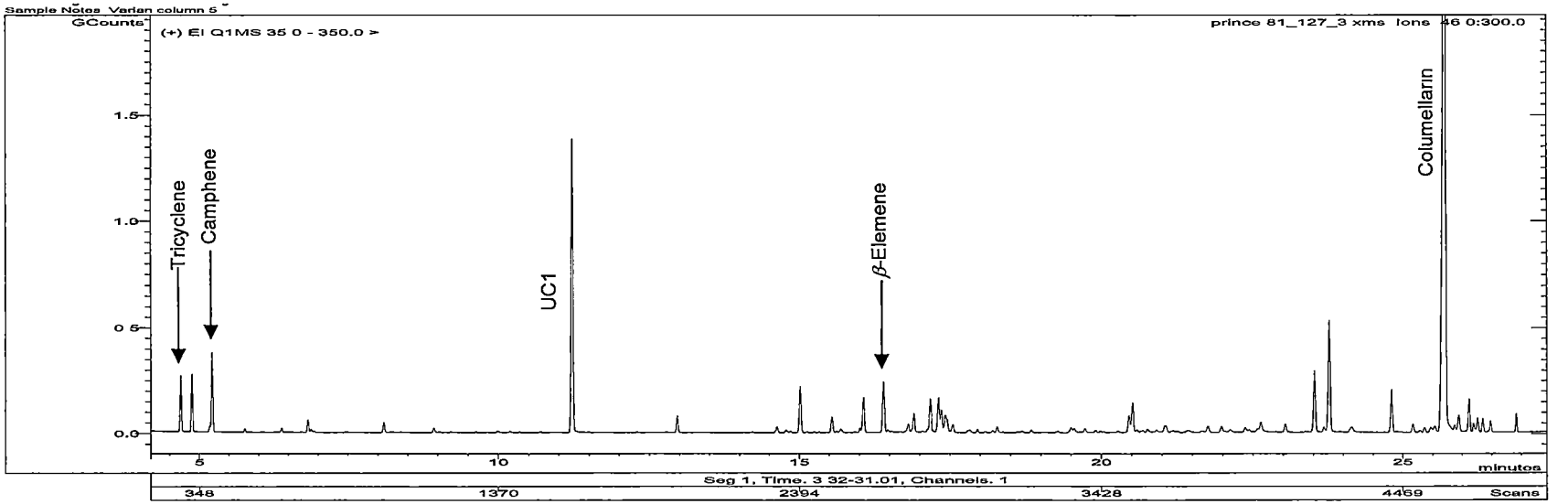


Figure 3.10 GC-MS chromatograms of the 'in vial' SPME samples of *C. oblonga* root.

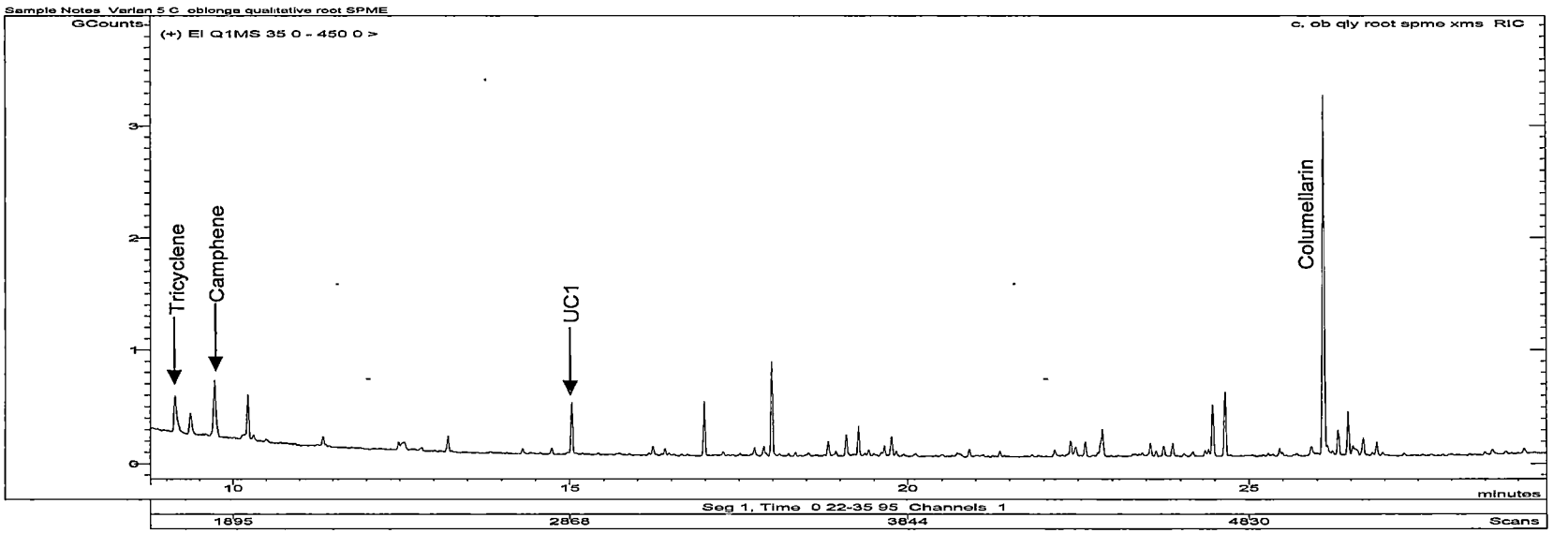


Figure 3.11 GC-MS chromatograms of SD oil of *C. oblonga* root

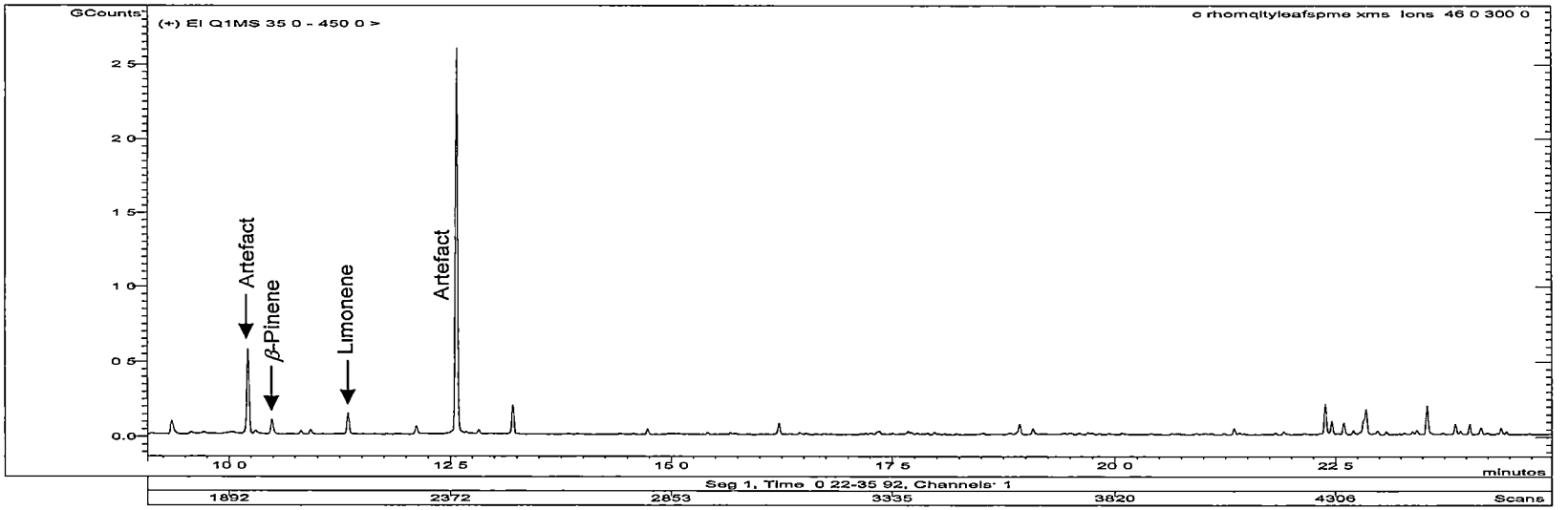


Figure 3.12 GC-MS chromatograms of 'in vial' SPME experiments of *C. rhomboidea* leaves

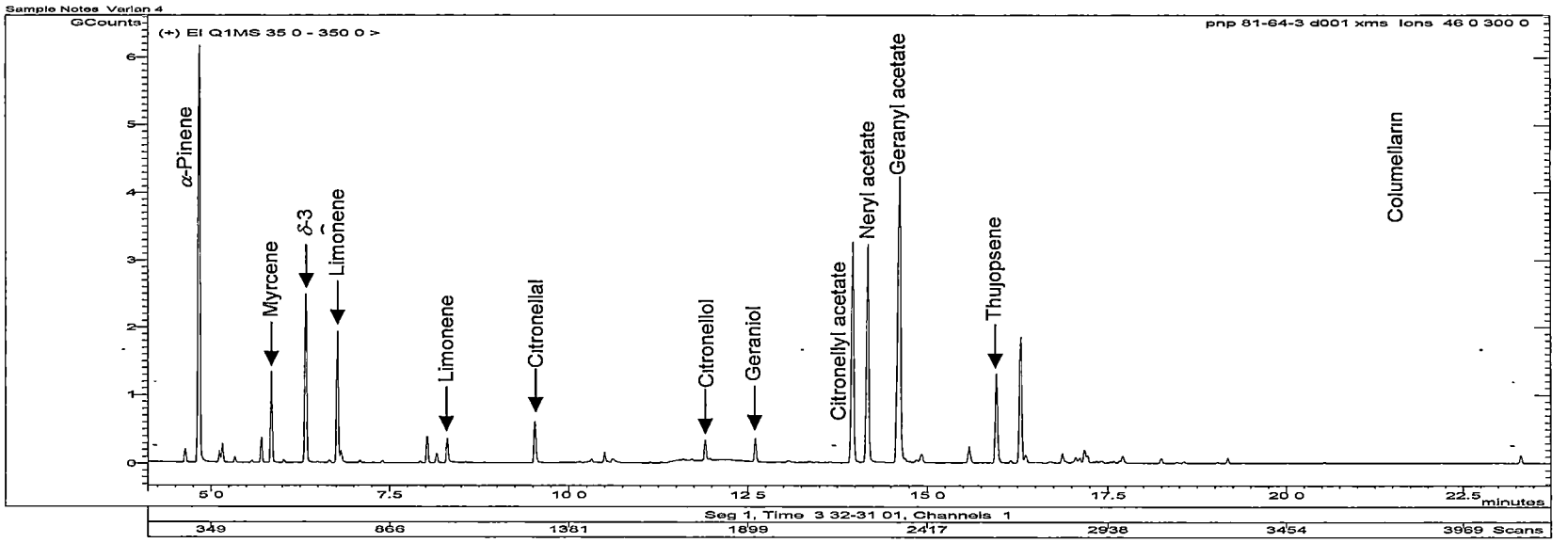


Figure 3.13 GC-MS chromatograms of SD oil obtained from of *C. rhomboidea* leaves.

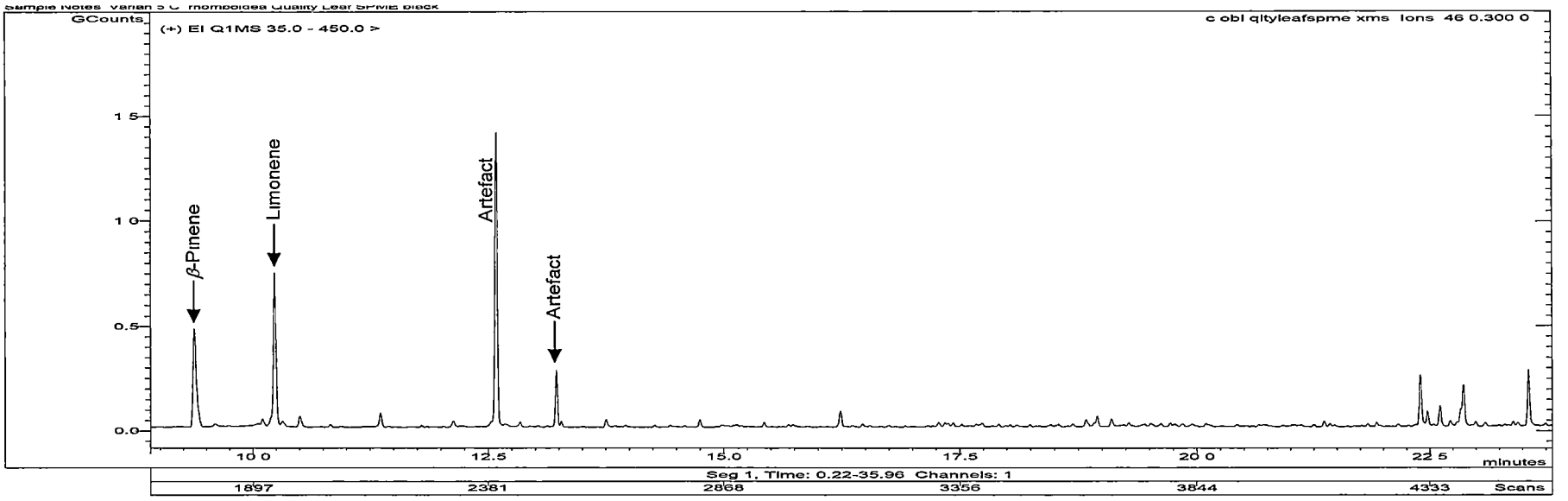


Figure 3.14 GC-MS chromatograms of 'in vial' SPME samples of *C. oblonga* leaves

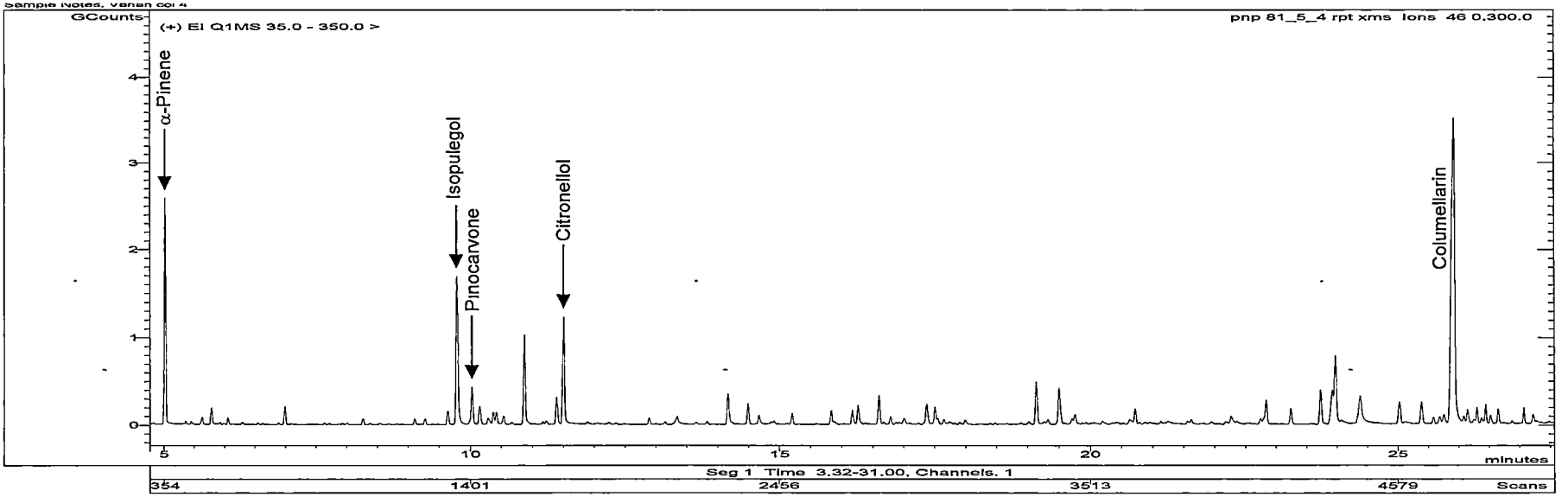


Figure 3.15 GC-MS chromatograms of SD foliage oils obtained from samples of *C. oblonga* leaves

According to work done by Zini and co-workers, (2001) on *Eucalyptus citriodora* leaves, an exposure time of 10 minutes was enough for the SPME fibre to adsorb sufficient plant volatiles to enable detection. The following compounds like α-pinene, citronellal, and citronellol were found in abundance (>10%) in *E. citriodora*. Although the *Callitris* leaves were exposed to the SPME fibre for 20 minutes, the release of major constituents of the leaves was not detected by GC-MS analysis. Thus it appears that *Callitris* leaves do not release significant quantities of volatiles into the atmosphere compared with their roots.

Terpenes are characteristic constitutive and inducible defence chemicals of conifers (Martin *et al.*, 2003). The peak period of monoterpene biosynthesis coincided with the time when the secretory cells of the glandular trichomes are metabolically active. The site of monoterpene biosynthesis in leaves has been localised to the secretory cells of glandular trichomes (Gershenzon *et al.*, 1989). Experiments performed on the leaves of *Mentha spicata* have shown that during the initial period of leaf development, the rate of monoterpene volatilisation was low (Gershenzon *et al.*, 2000). Plants, when subjected to pathogen or herbivore attack, activate biochemical defences. Oral secretions from feeding herbivore contain specific enzymes that provide the initial chemical signal that triggers the release of plant volatiles (Pare and Tumlinson, 1996). These factors could be a possible reason for the low rate of volatilisation from the leaves.

Identified terpenoids from roots and foliage of *Callitris* spp. analysed by GC-MS are presented in Table 3.1. All the samples represented are of 'in vial' experiments. The percentages represented in Table 3.1 are peaks on the SPME chromatogram and not percentages by weight within the plant since, SPME creates its own equilibrium for

each compound. This was the major difference between headspace SPME analysis and the analysis of neat oils. The constituents are arranged in the order of elution from the VF5-ms column. The constituents of the SD oils are the same as those presented in Table 2.2.

Table 3.1 SPME composition of volatile constituents released by roots and foliage as determined by GC-MS. Values are % composition of volatiles based on TIC integration.

Compound	Rt	*C. rhomboidea* roots	*C oblonga* roots	*C. rhomboidea* foliage	*C. oblonga* foliage
Tricyclene	9.13	5.3	4.3	-	-
α-Pinene	9.36	1.4	2.2	25.2	5.1
Camphene	9.72	8.9	6.0	-	5 5
β-Pinene	10 49	-	-	-	5 8
Myrcene	10.50	-	-	2.5	-
δ-3 Carene	10.92	-	-	-	1.5
Limonene	11.35	1.6	0.6	2 6	6 6
1,8 Cineole	11 40	0.6	-	-	-
α-Terpinolene	12 48	1.5	0.6	-	-
Isopulegol	13 75	-	-	1.6	-
Unknown compound 34	14 31	0.6	0 4	-	-
Unknown compound 1	15.03	5.6	3.5	-	-
Unknown compound 35	16.99	5.9	3.8	-	-
β-Gurjunene	17.83	1.9	-	-	-
β-Elemene	17.98	-	7.1	-	-
Unknown compound 36	18 35	0.6	0.6	-	-
Longifolene	18 43	12.4	-	-	-
Thujopsene	18.83	3.7	1 1	1.6	-
Unknown compound 7	19 10	9 8	1 7	-	-
Unknown compound 37	19 28	2 9	1.9	-	-
δ-Cadinene	19.34	12.0	0.5	-	-
α-Selinene	19.36	-	1.6	-	-
δ-Elemene	20.25	1 5	-	-	-
Spathulenol	20 50	0 4	-	-	-
Longiborneol	21 45	5 7	-	-	-
Columellarin	26 05	-	29 7	-	-

Unknown compound 34 180(45), 165(9), 148(42), 139(14), 125(25), 121(100), 120(28), 119(30), 112(24), 105(66), 97(12), 88(56), 80(10), 79(36), 77(39), 65(13), 44(54), 44(54), 43(21); **Unknown compound 35** 180(54), 165(10), 152(10), 149(15), 148(96), 137(9),

133(21), 121(47), 120(34), 119(30), 106(15), 105(46), 94(36), 93(36), 92(48), 91(41), 81(18), 79(47), 77(31), 68(100), 67(71), 59(12), 55(12), 53(32); **Unknown compound 36** (180(45), 165(14), 149(9), 121(100), 119(15), 105(57), 93(15), 91(25), 79(20), 77(20), 44(20) **Unknown compound 37** 180(34), 165(12), 149(9), 121(100), 105(47), 93(11), 91(22), 79(16), 53(5), 43(6).

3.3.2 Model experiment

In situ experiments were designed to analyse the viability of analysing the root volatiles released by intact *Callitris* plants. The soil probe designed and manufactured 'in house' was used to serve the purpose. The aim of this work was to define the detection limits for volatiles, such as 1,8-cineole, geranyl acetate, and major volatiles in SD root extract such as longiborneol in order to test the validity of this method for environmental release of volatiles from soil.

The model system was based on sand. A known amount of the volatiles (geranyl acetate, 1,8-cineole and *C. rhomboidea* SD oil) in solvent was distributed across the sand particles by rotary evaporation of the sand/terpene mixture to remove the solvent. The potential loss of volatile constituents during rotary evaporation of the sand/terpene mixture was a concern. Low pressure and gentle heating (25 °C) were used for the evaporation of solvent. GC-MS analysis of the terpene solution before rotary evaporation and a solution of the terpenes extracted from the sand/terpene mixture after rotary evaporation demonstrated no significant loss of the volatile constituents (Fig 3.16).

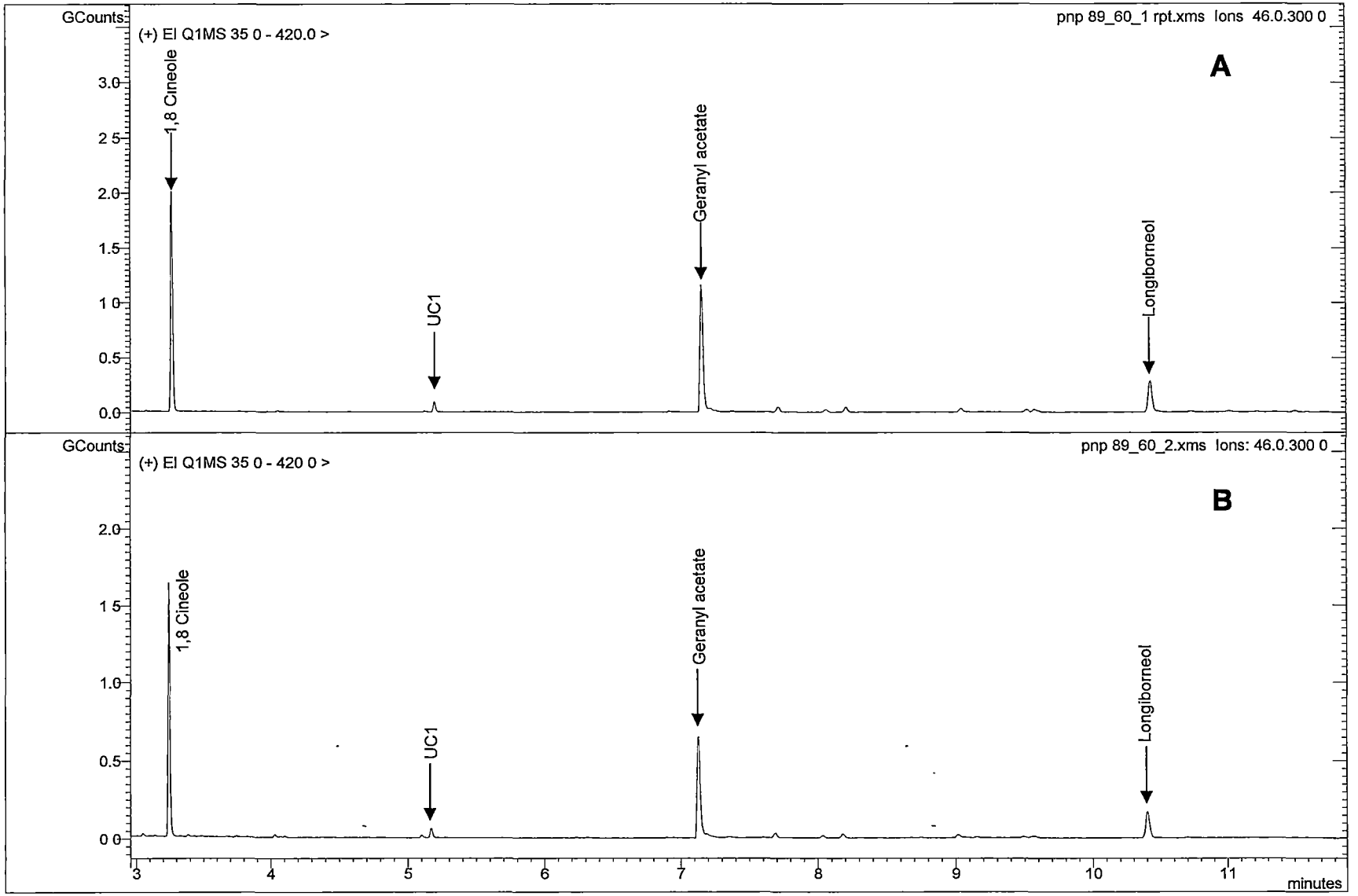


Figure 3.16 Comparison of GC-MS chromatograms of solvent samples of volatile constituents before (A) and after (B) rotary evaporation.

1,8- Cineole, geranyl acetate and the major *C. rhomboidea* root oil components such as UC1 and longiborneol, were used as marker compounds (Fig 3.16). However, only 1,8-cineole was detected from the SPME fibre by GC-MS of the sand/terpene mixture (Fig 3.17). The detection limit of 1,8-cineole was estimated to be 10 ppm (dilution 2).

According to Varian 320-MS GC-MS specifications, in Electron-Ionisation (EI) full scan mode, 1 picogram (pg) of sample of octafluoronapthalene in the SPME fibre, was enough for its detection by GC-MS. The higher detection limit for the terpene/sand mixture was either due to the adsorption of volatiles onto the sand (Ruiz *et al.*, 1998) or due to the absence of air movement between sand particles, thereby hindering the movement of volatiles into the headspace of the SPME fibre.

Inability of the GC-MS to detect geranyl acetate and oil components from the SPME fibre could be due to one or more of the following factors; the SPME fibre could be more efficient in detecting 1,8-cineole; geranyl acetate has a lower vapour pressure than 1,8-cineole, thereby less sample in the headspace; one ppm of a single component (1,8-cineole) was much easier to detect than 1 ppm of a mixture of oil components as is the case with the root oil, since all the components would give less than 1 ppm.

The 'model experiment' can not considered as an effective model for the *in situ* experiments since the detection limit of the volatiles from the model system was extremely high due to the above discussed factors.

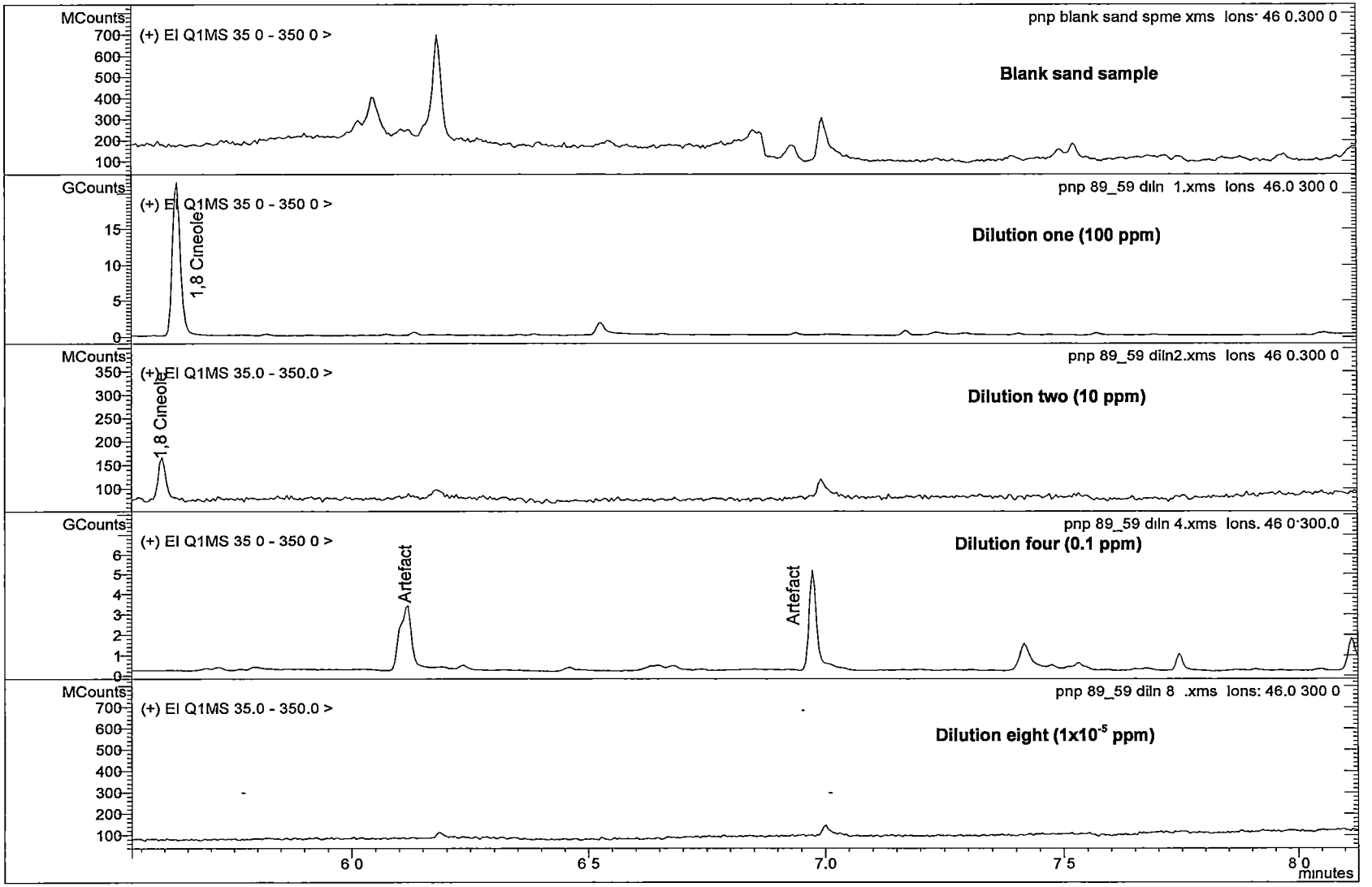


Figure 3.17 Comparison of the GC-MS chromatograms from the SPME analysis, using a soil probe, of different dilutions of a terpene mixture with sand.

3.3.3 In situ experiments

Before each sample collection the soil probe was cleaned by water/detergent and dried in an oven at 100 °C for 24 hours to remove volatiles adsorbed to its surface. The SPME fibre was desorbed in the GC each time before sample collection to prevent accidental contamination.

The soil probe was used to sample volatiles released from the roots of potted plants. Several volatiles were detected on analysing the GC-MS chromatograms from the SPME fibre of the potted plant roots. The major volatile that could be detected on analysing the GC-MS chromatograms of the *C. rhomboidea* potted plants was α-pinene. None of the major volatiles in SD oil or 'in vial' samples were detected following pot sample analysis. The chromatograms were scanned for *m/z* 180 and 121 ions, but none of the isomers were found.

The major volatiles identified from after SPME soil sampling of potted *C. oblonga* were cymene, cineole and limonene. These volatiles were not the characteristic components of the GC-MS chromatograms of the SD root oil of *C. oblonga*. These monoterpenes are the common constituents of conifers that are used in potting mixtures (Isidorov *et al.*, 2003). Columellarin which formed 30% of the total TIC of the oil was used as the marker compound. Columellarin was identified from the 'in vial' sample experiments (Fig 3.18).

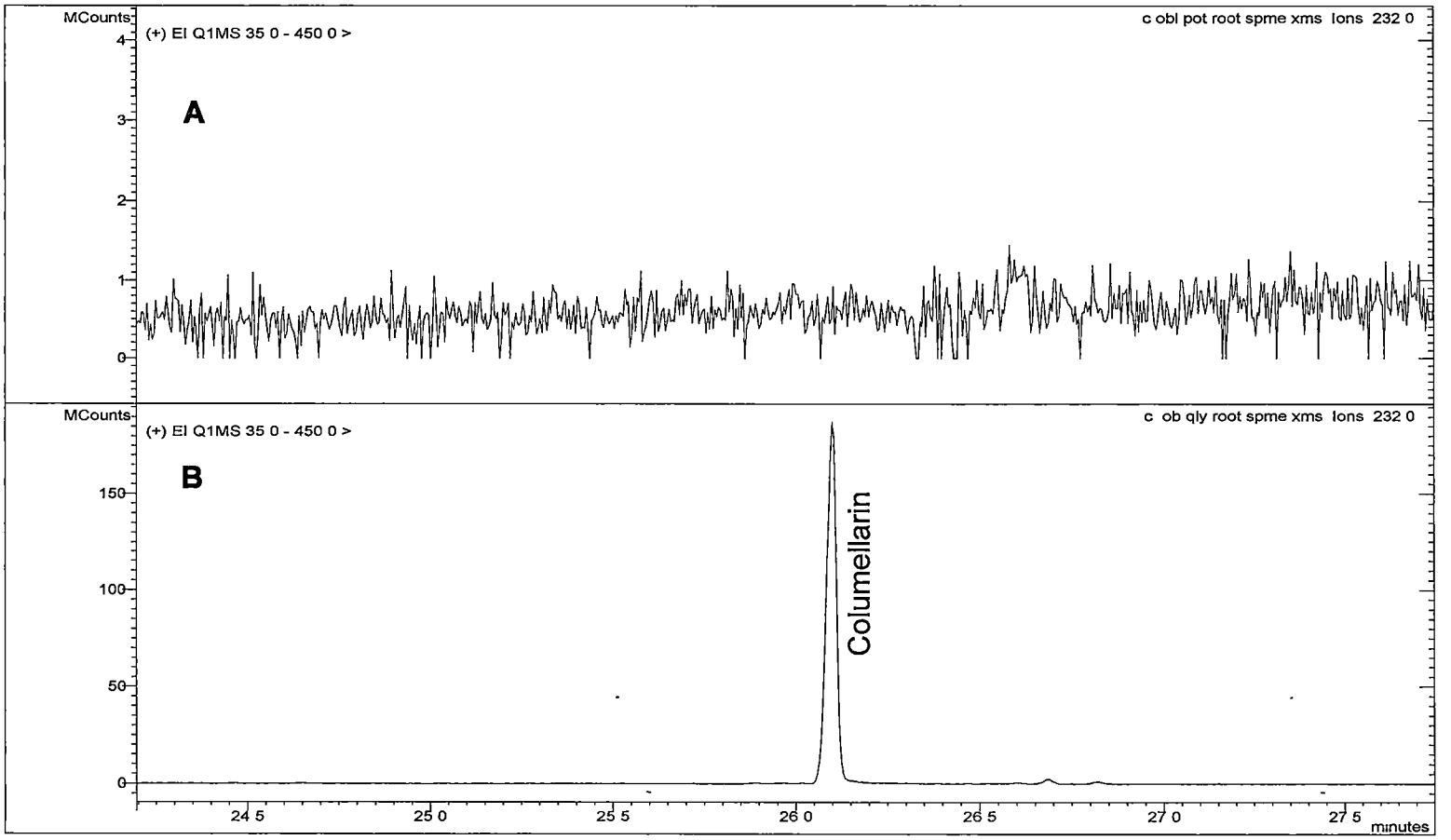


Figure 3.18 *C. oblonga* 'in pot' and 'in vial' samples scanned for *m/z* 232 ions.

Analysis of constituents from the *in situ* experiments of 'in ground' *C. rhomboidea* plants in two different locations in Tasmania by GC-MS analysis showed a large number of volatiles being evolved. GC-MS analysis of the first sample collected from the University of Tasmania campus showed no similarities between its volatile constituent profile and that of the SD root oil or the profile obtained from 'in vial' SPME experiments. The main compounds that were present in the sample were normal hydrocarbons (C_{15}-C_{20}) characteristic of petrochemical contamination. Silicon artefacts were also detected by the soil probe. It was assumed that hydrocarbons may have been washed by rain from vehicle exhausts or oil or bitumen from the nearby car park, and the soil might have become contaminated (Fig 3.19).

Unknown compound 1 which was the characteristic compound in the SD root oil of *C. rhomboidea* was used as the marker compound. As was previously described the GC-MS analysis of the 'in vial' sample of *C. rhomboidea* roots has shown the presence of 4 isomers of UC1. The GC chromatogram of the *in situ* sample was filtered for *m/z* 180 ions and 121 ions for detection of unknown MW 180 compounds. There was no detectable marker compound in the GC-MS trace of the *in situ* sample.

GC-MS analysis of the sample from the second collection site (Bellerive), demonstrated the presence of monoterpenes such as tricyclene, camphene and limonene (Fig 3.20). These monoterpenes were found in the same ratios as determined in *C. rhomboidea* SD root oil. Due to the presence of high levels of hydrocarbons from petroleum products these monoterpenes were a very small percentage of the total TIC of the sample.

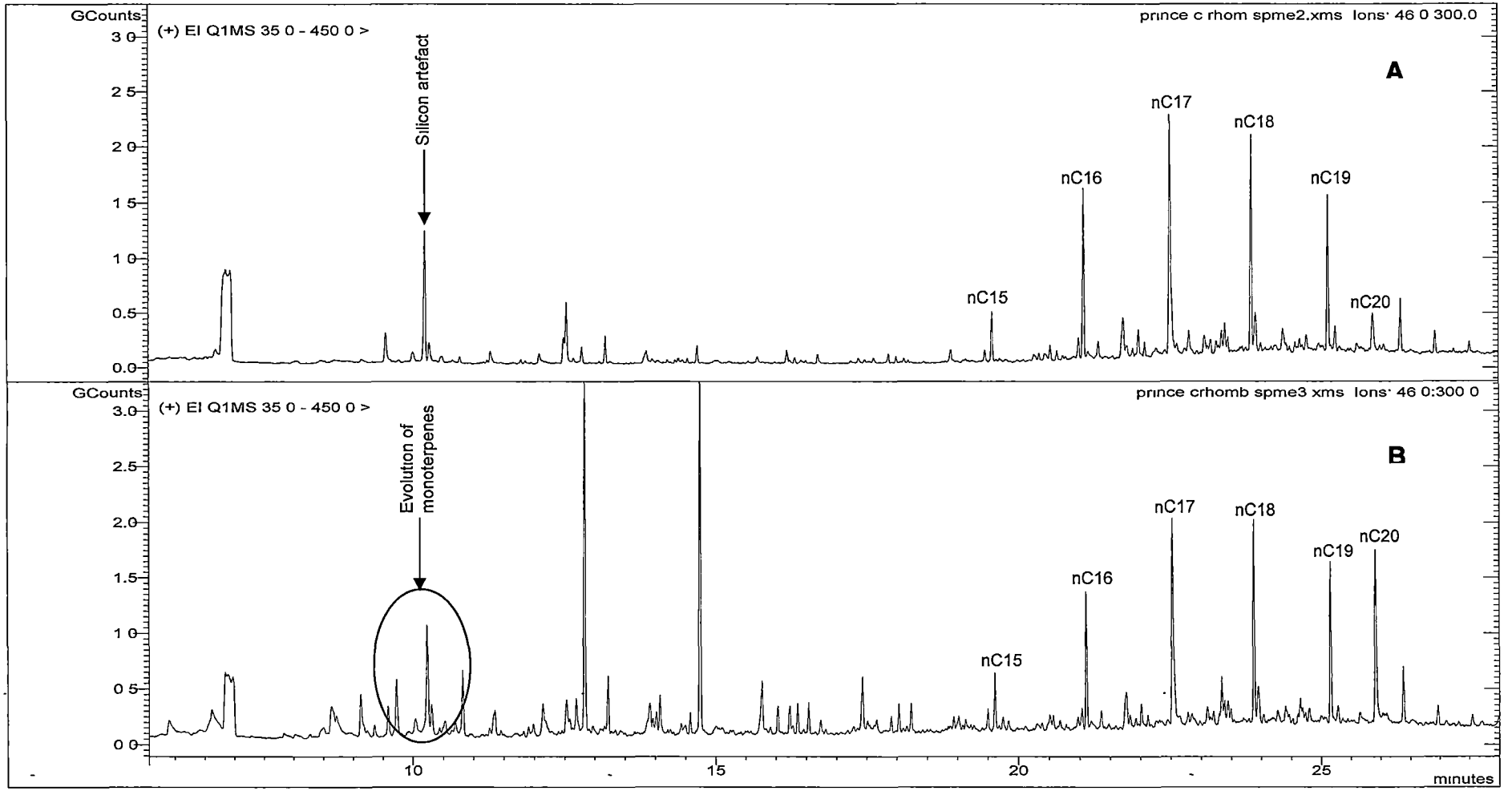


Figure 3.19 Comparison of GC-MS chromatograms of *in situ* SPME analyses of soil volatiles sampled in the vicinity of a *C. rhomboidea* growing at the University campus (A) and Bellerive (B).

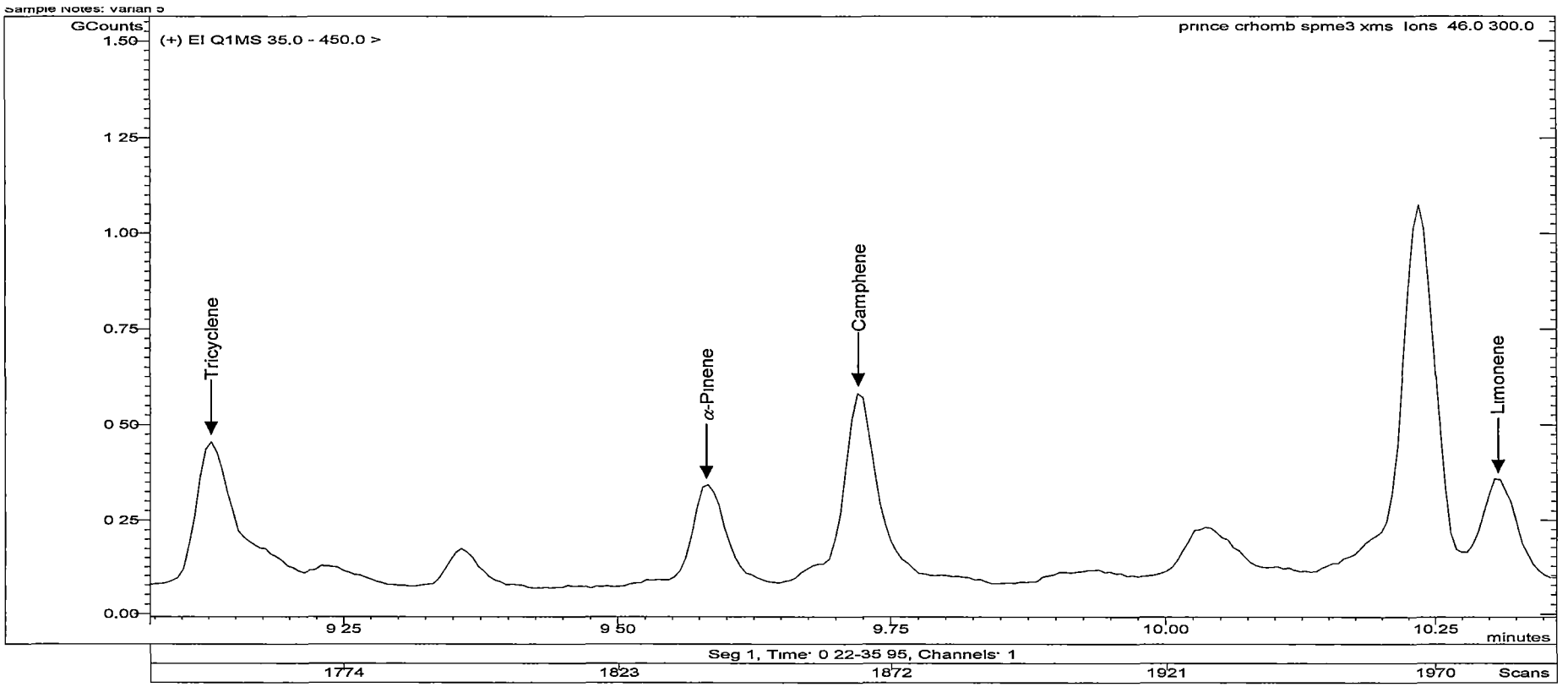


Figure 3.20 The GC-MS analysis of SPME sample obtained from Bellerive demonstrating the presence of monoterpenes.

These results suggest that the soil probe was efficient enough to detect the presence of volatiles released from the roots of the plant. None of the vast number of volatiles, especially sesquiterpenoids, detected during 'in vial' analysis of the *C. rhomboidea* roots could be detected from the intact plants. It is possible that the release pattern of these volatiles vary according to the conditions.

Studies done on other plants like *Arabidopsis* roots have shown that physical damage to the roots by herbivores or by other plant feedants could lead to evolution of plant volatiles (Van Poecke *et al.*, 2001).

'In vial' experiments demonstrated that damage to the root structure leads to the release of leaf volatiles detectable by SPME. It could be assumed that the difference in the emission of volatiles during stress could be due to the expression of different genes (Gatehouse, 2002). According to work done by Zini and co workers in 2001, volatiles released could vary with the time of the day and climate. Volatile emission acts as a defensive mechanism against high temperature damage. Volatile constituents in the roots are stored in specialised secretory structures such as glandular trichomes or resin ducts (Gershenzon *et al.*, 2000).

Root exudates may regulate the soil microbial community in their immediate vicinity; help the plant cope with herbivores by acting as toxins or feeding deterrents; change the physical and chemical properties of the soil; and could inhibit the growth of competing plant species and communicate with other species (Nardi *et al.*, 2000; Park *et al.*, 2002). Chemical volatiles released from injured plants not only affect the herbivore growth but also can cause harm to the neighbouring plants by triggering defence responses (Arimura *et al.*, 2002).

These data support the view of Langenheim (1994), that oils are released into the soil following damage by herbivores or other predating animals.

3.4 CONCLUSION

The 'soil probe' could be used as a new method for analysing the release of volatile constituents from underground parts of a plant. The experiments demonstrated that *Callitris* roots released volatiles to the surroundings from stressed as well as unstressed roots. The number of volatiles released from the roots in a stressed condition was more than in the unstressed state.

CHAPTER 4

Antimicrobial Screening of Essential Oils, Fractions and Extracts of *Callitris* spp. Plants

4.1 INTRODUCTION

The antimicrobial action of essential oils is well known with many reports in the literature (Chorianopoulos *et al.*, 2006). SD oils obtained from *Callitris rhomboidea* and *Callitris oblonga* plants have never been a subject of study for their antimicrobial properties.

The best known essential oil from the *Callitris* spp. is Australian blue cypress oil obtained by steam distillation of the wood of *C. intratropica*. The plants are cultivated and the oil is produced by Australian Cypress Oil Pty Ltd (ACO).d About 3 000 hectares of Northern Territory plantations are managed by ACO and this is the only commercially viable plantation in the world (The Australian Cypress Oil Propriety Limited, 1995). The Aboriginal Pharmacopeia states that aborigines used the decoction of *C. intratropica* bark in water as an abdominal wash to relieve abdominal cramps. The wood ashes were mixed with water and smeared over the affected part of the body to relieve minor aches and pains (Barr *et al.*, 1988).

Australian blue oil has an aroma similar to sandalwood oil (*Santalum album*), oil of guaiac wood (*Bulnesia sarmienti*), and oil of vetiver (*Vetiveria zizanioides*). Therefore it is marketed in applications and proportions in perfumes, cosmetic, and body care products, in a similar manner to the sandalwood and vetiver oils (The Australian Cypress Oil Propriety Limited, 1995). It was determined that the blue

colour of the oil was due to the presence of azulenes such as guaiazulene and guaiol (Collins, 2000).

We hypothesised that the roots of the *Callitris* spp. plants may release the volatile constituents into the soil and surrounding areas to produce a protective antimicrobial or antifungal effect. The aim of my study was to evaluate the antimicrobial activity of *C. rhomboidea* and *C. oblonga* foliage and root derived essential oils against a diverse panel of microorganisms.

4.1.1 Selection of microorganisms for the study

The selection of microorganisms for susceptibility testing was based on the precedent studies done on Myrtaceous oils and the basis of our hypothesis that the root oils may have a biological role in protecting the plant against fungal organisms.

The bacterial organisms tested for the susceptibility study included Gram positive organisms such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Bacillus subtilis* and *Dermatophilus congolensis* (causative organism for pustular dermatitis); and Gram negative infection causing organisms such as *Proteus mirabilis, Escherichia coli* and *Pseudomonas aeruginosa.*

The fungal organisms selected for susceptibility study included a filamentous fungus, *Candida albicans*, an indicator organism for the potential activity against yeasts; dermatophytes such as *Epidermophyton* spp., *Trichophyton* spp. and *Microsporum* spp., the causative organisms for dermatophytosis; and a plant fungus *Phytopthora infestans*, a causative organism of the root rot that mainly affects perennial woody plants (Invasive species fact sheet, 2004).

4.2 MATERIALS

4.2.1 General

The incubation of the bacterial and fungal organisms was done at 25 °C and 37 °C using incubators (Qualtex) supplied by Watson Victor Ltd (Melbourne, Australia). Pipetting was performed using pipettes of various sizes (100, 200, 500 and 1000 µl) supplied by Gilson Instruments (West Grove, USA) and multichannel pipettes (50 µl) supplied by Titertek Instruments (Huntsville, USA). Microtitre plates for determining minimum inhibitory concentration (MIC) were flat-bottomed 96- well polystyrene plates supplied by Iwaki Co., Ltd (Tokyo, Japan). Petri dishes for the susceptibility studies were supplied by Biolab (Melbourne, Australia). Vortexing was done with the help of a vortex mixer (250 VM) bought from Thermoline Scientific (Melbourne, Australia). The UV spectrophotometer, model Spectronic 20, was from Bausch & Lomb Pty. Ltd (Melbourne, Australia).

Column chromatography was performed using glass columns (diameter 25mm and 40 mm), on silica gel (grade 923, W.R Grace and Co., Maryland, USA) (13 cm and 19 cm column packed height). Solvents used in the experiments were dichloromethane (DCM) LR grade (BDH, Melbourne, Australia); methanol LR (98%) (Ajax Finechem, Sydney, Australia); hexanes AR (BDH, Melbourne, Australia) and petroleum spirits (BDH, Melbourne, Australia). Solvents were freshly redistilled before use. Thin layer chromatography was performed on Merck Kieselgel DC-Alufolien 60 F254 plates from Sigma-Aldrich (Sydney, Australia). The plates were viewed under a UV light at 254 nm, Chromato-Vue Model CG-20, supplied by Ultraviolet products Inc (CA, USA).

4.2.2 Bacterial and Fungal isolates

Bacterial organisms used for the susceptibility testing included the Gram positive organisms such as *Staphylococcus aureus* (American Type Culture Collection (ATCC) 6538), *Bacillus cereus* (ATCC 1778), *Bacillus subtilis* (ATCC 6633), *Staphylococcus epidermidis* (School of Medicine, University of Tasmania, Hobart, Australia) and *Dermatophilus congolensis* (Department of Agriculture, Perth, Australia); Gram negative organisms *Proteus mirabilis* (ATCC 43071), *Escherichia coli.* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 27853).

Eight isolates,one each of *Epidermophyton floccosum, Microsporum canis, Microsporum gypseum, Trichophyton rubrum* (downy strain), *Trichophyton rubrum* (granular strain), *Trichophyton mentagrophytes* (var. interdigitale), *Trichophyton soudanense,* and *Candida albicans* (ATCC 6505) were obtained as a gift from the culture collection of Assoc Prof. David Ellis, Mycology Unit, Women's and Children's Hospital , North Adelaide, South Australia. *Phytopthora infestans* was obtained from the culture collection of the School of Agricultural Science, University of Tasmania, Hobart, Australia.

4.2.3 Essential oils

Retail samples of essential oils screened in this study included kanuka oil (*Kunzea ericoides*), manuka (*Leptospermum scoparium*), tea tree (*Melaleuca alternifolia*), niaouli A (*M. viridiflora*), niaouli B (*M. quinquenervia*), Australian blue cypress oil (*C. intratropica*) and cajuput oil (*M. leucadendron*). Niaouli oils A and B, and cajuput oil were supplied by Gould's Pharmacy (Hobart, Australia); kanuka and manuka oil were obtained from Marrickville Estate (Sydney, Australia); tea tree oil was produced by Felton Grimwade and Bickford Pty, Ltd (Melbourne, Australia); and *C.*

intratropica oil was produced by The Australian Cypress Oil Company Pty Ltd (Sydney, Australia).

The oil samples were analysed by GC-MS (as described in Chapter 2) to verify their composition. Leaf and root steam distilled oils as well as solvent extracts from different organs from *C. rhomboidea* and *C. oblonga* plants were obtained as is described in Chapter 2. Nerol, geraniol, citronellol and geranyl acetate, of purity ≥99%, were bought from Sigma Chemicals (Sydney, Australia).

4.2.4 Antimicrobial discs and pharmaceutical products

Blank paper discs of 6 mm in diameter were used for analysing the activity of essential oils. Antimicrobial discs used for positive controls of antibacterial screening were ampicillin (10 μg), vancomycin (5 μg), trimethoprim (5 μg), penicillin (5 IU), gentamycin (10 μg), ciprofloxacin (5 μg), and streptomycin (10 μg). All the discs were supplied by Oxoid (Adelaide, Australia).

4.2.5 Proprietary antifungal drugs

Antifungal agents used as positive controls were Nilstat (Sigma Pharmaceuticals Pty Ltd, Melbourne, Australia), the active ingredient being nystatin (100 000 units of nystatin/ml); Nizoral (Janssen-Cilag Pty Ltd, Sydney, Australia), active ingredient being ketoconazole (2mg/g); and Lamisil (Novartis Pharmaceuticals Australia Pty Ltd, Sydney, Australia) the active ingredient being terbinafine hydrochloride (10mg/g).

4.2.5 Media

The media used for the antimicrobial assays are shown in Table 4.1. Mueller-Hinton agar (MHA) or brain heart infusion agar (BHA) and Mueller-Hinton broth or brain

heart infusion (BHI) were used as a medium for the susceptibility testing of bacteria. Sabouraud dextrose agar and RPMI-1640 were used as the media for the susceptibility testing of fungi. All the media used for the assays were supplied by Oxoid (SA, Australia). RPMI-1640, morpholinosulfonic acid (MOPS) and Tween 80 were supplied by Sigma Chemicals (Sydney, Australia).

Table 4.1 Media used for the antimicrobial and antifungal assay.

Media	Mass of powder (g)	Volume of distilled water (L)	Supplements	pH
Mueller-Hinton agar (MHA)	38.0	1.0	Tween 80 (0.001 v/v %)	7.3±0 1
Mueller-Hinton broth (MH)	22.0	1 0	Tween 80 (0.001 v/v %)	7 3±0.1
Sabouraud dextrose agar (SDA)	65.0	1 0	Tween 80 (0 001 v/v %)	5 6±0 1
RPMI-1640 with L-glutamate, without bicarbonate	10.4	1 0	Tween 80 (0.001 v/v%), Glucose (0.2 w/v%), MOPS (As necessary)	7 0±0.1
Brain Heart Infusion broth (BHI)	37.0	1.0	Tween 80 (0.001 v/v %)	7.3±0 1
Brain Heart Infusion agar (BHA)	47.0	1.0	Tween 80 (0.001 v/v %)	7.3±0.1
Saline	8 5	1 0	None	7.0±0 1

3 INSTRUMENTS

4.3.1 Gas Chromatography- Mass Spectrometry (GC-MS)

Oil samples and solvent extracts diluted in hexane (5 mg/ml) were analysed by GC-MS as is described in Chapter 2.

4.3 CULTURE PREPARATION

4.3.1 Bacterial culture preparation

All isolates were stored at -70° C on MHA until required except *D. congolensis* which was prepared on BHA. Overnight broth cultures were prepared by inoculating 15-20 ml MH broth with 1-2 colonies taken from a MHA plate or BHA plate for *D. congolensis*, followed by incubation at 37 °C for 24 hours. For susceptibility testing the optical density of the bacterial suspensions were adjusted to 80% transmission at

500 nm, which corresponded to the 0.5 McFarland standard (1 x 10^8 colony forming units(cfu)/ml). In addition the suspension was visually compared with the 0.5 McFarland standard against a card with a white background and contrasting black lines. Final inocula concentrations were confirmed by using Miles-Misra viable counts (Al-Jabrah *et al.*, 2007).

4.3.2 Fungal inocula preparation

Dermatophytes and *Ph. infestans* were grown on SDA slopes at 25 °C for 7 days. Seven-day-old colonies were flooded with 0.85% saline. Fungal growth was gently probed with a Pasteur pipette the resulting mixture of conidia and sporangiospore and hyphal elements was withdrawn and transferred to a sterile tube and vortex mixed for 3-5 minutes. After heavy particles were allowed to settle, the upper homogeneous suspension was collected. The density of the conidial suspension was read and adjusted with 0.85% saline to an OD that ranged from 70-72% transmittance at 520 nm. This suspension corresponded to approximately 0.4 x 10^4 to 5 x 10^4 cfu/ml.

C. albicans inocula were prepared by the procedure described above, except the incubation time was 48 hours and the incubation temperature was 37 °C. The final fungal inocula concentrations were confirmed by Miles-Misra viable counts (NCCLS, 2006; NCCLS, 1997; NCCLS, 1998).

4.4 ANTIMICROBIAL ASSAYS

The disc diffusion and broth microdilution assays were based on reference methods M7-A6, M27-P and M-38P recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for bacteria, yeasts and conidium forming filamentous fungi respectively (NCCLS, 2006; NCCLS, 1997; NCCLS, 1998). The only modification was the addition of Tween 80 at a concentration of 0.001% v/v.

4.4.1 Agar gel diffusion assay

A solid medium diffusion technique using sterile paper discs was used for screening the antibacterial activity of the essential oils. For this, 1ml of the bacterial suspension was uniformly spread on MHA plates. After the inoculum suspension was absorbed into the agar, paper discs were placed onto the agar plates. Ten microlitres of essential oils or 10 mg of petroleum spirit/methanol extracts were pipetted onto each paper disc. Paper discs containing extracts were evaporated off to remove the solvents. Plates were sealed with parafilm. The system was incubated at 37 °C for 24 hours. At the end of the incubation period, the bacterial zone of inhibition (ZI) was measured as excess radius from the edge of the disc in mm. It was considered as positive antibacterial activity when observed growth ZI excess radii were equal to or greater than 1 mm. Controls included in this assay were commercial essential oils, antibiotic discs and untreated plain discs.

Antifungal activity of the essential oils was also determined in the same manner as described above but on SDA plates and with an incubation time of 7 days at a temperature of 25 °C for dermatophytes or 48 hours at 37 °C for *C. albicans*. The controls were commercial oils and proprietary antifungal preparations. Zone of inhibition was measured at 3 and 7 day intervals for all dermatophytes and plant fungi; readings at 1 day and 2 day were performed for *C. albicans*.

4.4.2 Broth microdilution assay

Microtitre trays contained a series of doubling dilutions of the *C. rhomboidea* leaf oil and its components (geraniol, citronellol, and nerol) in 100 µl of growth medium RPMI 1640. The essential oil and its components were tested in the range of 8% to 0.008% v/v in the final inoculum mixture. Inoculation was done with 100 µl of the

fungal (*M. canis*) inoculum prepared as described above. One well served as a growth control, containing only 100 µl media (RPMI 1640) and 100 µl inoculum.

Test plates were incubated at 25 °C for 7 days. After incubation MIC's and MFC's were determined. The MIC's were determined visually with the aid of a reading mirror as follows. Growth in each well was compared with control and was scored numerically as follows: 4, no reduction in growth; 3, approximately 75% of the growth control; 2, approximately 50% of the growth control; 1, approximately 25% of the growth control; 0, optically clear or no visible growth (NCCLS, 1998). The MIC was determined as the lowest concentration of agent corresponding to a 75% reduction in growth, compared to the control (Espinel-Ingroff *et al.*, 1994).

MFC's were determined for each sample, by subculturing 10 µl from wells showing no visible turbidity and spot inoculating onto SDA plates. MFC's for *M. canis* were determined as the lowest concentration resulting in no growth in the subculture.

4.5 FRACTIONATION OF C. RHOMBOIDEA LEAF OIL

Fractionation of *C. rhomboidea* foliage oil was performed using flash column chromatography on silica gel. Initially a 25 mm glass column was used, packed to a height of 13 cm. A larger scale fractionation (2.4 g of oil) was performed using a 40 mm diameter column packed to a height of 19 cm. The oil was eluted with the following solvents: petroleum spirits (100%); petroleum spirits/dichloromethane 80:20, 60:40, 40:60, 20:80, 10:90; dichloromethane (100%); dichloromethane/ethyl acetate 80:20, 60:40, 40:60, 20:80, 10:90 and ethyl acetate (100%). Two bed volumes (200 ml) of each solvent were used.

Fractions were analysed by TLC and those showing spots at the same Retention factor (RF) value were combined appropriately. Excess of solvents was removed by rotary evaporation. The fractions containing various series of compounds (hydrocarbons, actetates, ethers, ketones, alcohols) were analysed and identified by GC-MS. The activities of the fractions against *M. canis* were determined using the disc diffusion technique.

4.6 RESULTS AND DISCUSSION

4.6.1 GC-MS analysis of the commercial oils

The volatile composition of oil obtained from *M. viridiflora*, *L. scoparium*, *M. alternifolia*, *M. leucadendron*, *K. ericoides*, *M. quinquenervia*, and *C. intratropica* are given in Table 4.2. Table 4.2 represents the relative amount of individual components of the oil expressed as percentage peak area relative to total ion current (TIC) of the whole oil.

Monoterpenes accounted for about 86-96% in *M. viridiflora*, *M. alternifolia*, *M. leucadendron* and *M. quinquenervia;* while sesquiterpenes varied between 2-10%. The major monoterpenes included 1,8-cineole varying from as low as 6% in *M. alternifolia* to 80% in *M. leucadendron.* Other monoterpenes included in all the *Melaleuca* spp., included terpinene-4-ol (0.1-37%), α-terpineol (1.3-24.3%), linalool (0.1-17.7%), limonene (1.3-7.3%), α-pinene (1.6-7.1%); while γ-terpineol (24.3%) was found only in *M. viridiflora.* The major sesquiterpenes included aromadendrene (0.2-2.3%), and caryophyllene (0.2-1.4%). These results were in agreement with the published works by Moudachirou *et al.*, (1996) (*M. quinquenervia*); Ramanoelina *et al.*, (1992) (*M. viridiflora*); Swords and Hunter, (1978) (*M. alternifolia*) and Ekundayo *et al.*, (1987) (*M. leucadendron*).

Table 4.2 Composition of essential oils obtained from different organs of the plants as determined by GC-MS (percentage of total ion current). Peaks are listed in elution order from the VF5-ms column.

Compound	KI	*Melaleuca viridiflora*	*Leptospermum scoparium*	*Melaleuca alternifolia*	*Melaleuca leucadendron*	*Kunzea ericoides*	*Melaleuca quinquenervia*	*Callitris intratropica*
α-Thujene	926	0.1		1 0	0 3	0.5	0.1	
α-Pinene	935	1.6	2 7	3.3	5.9	46 7	7 1	0.7
α-Fenchene	951							
Camphene	953	0.1			0.1			
Benzaldehyde	967				0 1		0.1	
Sabinene	974	1.6		0.7	2.2		2 7	
β-Pinene	981	0.7	0 1	0.6	0.4	0.4	0 2	
Myrcene	988							
α-Phellandrene	1007			0 4				
δ-3-Carene	1008							0.3
α-Terpinene	1011			9.0				
1,4-Cineole	1017	0 2						
p-Cymene	1028	1 9	0.3		2.0	7.9	1.2	0.1
Limonene	1033	2 9	0.1	1.3	4 9	1 0	7.3	0.1
β-Phellandrene	1034			0.5				
1,8-Cineole	1037	37.1	0.2	5.8	80.2	5.7	41.9	
γ-Terpinene	1062		0.1	18.6			0.2	
Terpinolene	1089			3 1				
Cymenene	1094							0 1
Linalool	1102	2 3	0 1		1.1	1.3	17.7	
α-Pinene oxide	1107							
exo-Fenchol	1126	0.3						
α-Campholenal	1134					0.3		
cis-Limonene oxide	1139							
1-Terpineol	1141	0.4						
trans-Pinocarveol	1149	0.2				0 4		
cis-Verbenol	1153					0.3		
Camphor	1156	2.5						
cis-β-Terpineol	1174	1.3						
Borneol	1180	0 3						
Terpinen-4-ol	1187	0 4	0.1	36 9	0.1	0.1	0.9	

Table 4.2 continued...

Compound	KI	*Melaleuca viridiflora*	*Leptospermum scoparium*	*Melaleuca alternifolia*	*Melaleuca leucadendron*	*Kunzea ericoides*	*Melaleuca quinquenervia*	*Callitris intratropica*
Cryptone	1196							
α-Terpineol	1203	24.3		3 4	1 3	0 8	9 9	0.1
Myrtenal	1204							0 1
γ-Terpineol	1207	7.8						
Verbenone	1217					0 2		0.2
Trans-carveol	1226					0 3		
2-Hydroxycineole	1233	0 2						
Bornyl acetate	1289							
Terpinyl acetate	1348	0.5					0 5	0 3
α-Cubebene	1349		2 9			0.3		
Myrtenic acid	1357							1.3
α-Copaene	1379		6 5	0.2		1.0		
β-Elemene	1392		0.5					2.0
Methyl eugenol	1401	0.3						
α-Gurjunene	1410	0 9	0.8	0.4		0.3		0.6
Caryophyllene	1424	0 2	1 4	0.3			1.0	0 1
8-Hydroxycarvotanacetone	1434	0.7						
α-Guaiene	1437							3.2
Aromadendrene	1441		2.3	1.4		0.8	0.2	
α-Humulene	1460						0.2	
Allo-Aromadendrene	1464	0.3	0.7	0.5		0 6	0.2	
Unknown compound 33	1477							7 1
Mixed sesquiterpenes	1491							5.0
β-Selinene	1494		5 9					5.7
Viridiflorene	1494			1.2	0.1			
Mixed Sesquiterpenes	1495					1.1	0 2	
Bicyclogermacrene	1499			0.4				
α-Selinene	1500		5.2					5.4
δ-Guaiene	1505							2.8
δ-Cadinene	1522		4.4	1 0				
1S trans-Calamenene	1526		12 9			6 2		
Cadina-1,4-diene	1538		3 8	0.1		0 1		
Flavesone	1539		7 0			0.4		
α-Calacorene	1547		0 9					

Table 4.2 continued...

Compound	KI	*Melaleuca viridiflora*	*Leptospermum scoparium*	*Melaleuca alternifolia*	*Melaleuca leucadendron*	*Kunzea ericoides*	*Melaleuca quinquenervia*	*Callitris intratropica*
Elemol	1552							1.7
trans-Nerolidol	1562					1.5	4.8	
Palustrol	1577					0 9		
Spathulenol	1584		1.0	0.1		1 8		
Caryophylline Oxide	1590		1 0				0 4	
Globulol	1594		0.5	0.2		0.3	0.1	
Guaiol	1603							12.7
Viridiflorol	1603		1.0	0.1		8 3	1 3	
Ledol	1613					2 8	0 3	
Isoleptospermone	1615		6.8					
Cedrol	1620							
Leptospermone	1625		19.1			2.2		
γ-Eudesmol	1638							7.8
β-Eudesmol	1664							11 3
Bulnesol	1672							7.8
Dihydrocolumellarin	1900							5.4
Total		89 6	88.3	90.5	98.8	94 7	98.5	80.2

As reported by Porter and Wilkins (1999), commercial leaf oils of *Leptospermum scoparium* (manuka) contained low levels of monoterpenes (≤ 3%) while sesquiterpene hydrocarbons accounted for about 60%. Interestingly oxygenated sesquiterpenes (calamenene) and triketones (flavasone, isoleptospermone and leptospermone) accounted for about 30% of the oil, of which the triketone-containing fraction was responsible for most of the antimicrobial activity of the oil. *K. ericoides* (kanuka) oil was characterised by high levels of monoterpenes (66%); α-pinene (46.7%) being the most prominent. Sesquiterpenes accounted for about 28%. The major sesquiterpenes were viridiflorol (8.3%), *trans*-calamenene (6.2%), and ledol (2.8%).

Out of the 80% of the total composition of the commercial wood oil of *C. intratropica*, 1.7% was monoterpenes while 78.3% of the oil was sesquiterpenes. The major sesquiterpenes included α and β eudesmols (19.1%), guaiol (12.7%) and α and β selinenes (11.1%).

4.6.2 Antimicrobial assays

4.6.2.1 Antimicrobial activity

Antimicrobial activities of *Callitris* spp. essential oils; commercial oils and antibiotic discs are summarised in Table 4.3.

Table 4.3 Zone of inhibition (ZI) as excess radius from the susceptibility disc for essential oils from different organs of *Callitris* spp., commercial oils and standard antibiotic discs versus a range of bacteria

Samples	Quantity of agent applied to disc	*S. epidermidis* (ATCC 12315)	*P mirabilis* (ATCC 43071)	*B. cereus* (ATCC 11778)	*E. coli* (ATCC 8739)	*B. subtilis* (ATCC 6633)	*P. aeruginosa* (ATCC 27853)	*D. congolensis*
		ZI (mm)	ZI (mm)	ZI (mm)	ZI (mm)	ZI (mm)	ZI (mm)	ZI (mm)
C oblonga leaf oil	10 µL	2.0	0.0	2 0	3 0	3.5	0 0	0 0
C. rhomboidea leaf oil	10 µL	2.0	2.5	2 0	2 0	2.0	1.0	2.0
C oblonga root oil	10 µL	1.0	1 0	2 0	0 0	3.0	0 0	1 0
C. rhomboidea root oil	10 µL	2.0	2 0	2 0	0. 0	2 5	0 0	0 0
M. alternifolia	10 µL	5.0	3 3	2 0	3.0	1.5	0 0	1.0
K.ericoides	10 µL	12.0	1.0	2.5	1.0	3.5	1 0	3 0
L scoparium	10 µL	16 0	6 0	7.5	1.5	11.5	0.0	9.0
M. leucadendron	10 µL	3 0	2 5	1.0	2 5	6.0	0 0	2 5
M. Viridiflora	10 µL	4 0	1.5	3.0	4 0	4 0	0.0	4 0
M. quinquenervia	10 µL	2.0	2.5	3 0	3.0	2 5	0 0	1.0
C intratropica	10 µL	2.0	0 0	1 5	0.0	2.5	0 0	2.0
Ampicillin	10 µg	1.0	6.5	0.0	3.0	0.0	3.0	3 0
Trimethoprim	5 µg	2.0	0 0	0.0	0.0	11.5	0 0	1 5
Penicillin G	5 IU	0.0	3 5	0.0	2 0	0.0	1 0	0.0
Gentamicin	10 µg	9.0	5.5	9 5	5 0	10 0	3 0	8.0
Ciprofloxacin	5 µg	7.0	0.0	11.0	11 5	14.0	0.0	10.5

Callitris spp. oils exhibited some activity against all of the microorganisms except *P. aeuroginosa. C. rhomboidea* leaf oil, was the most potent among all the *Callitris* oils. Although on comparing the activity of the *Callitris* oils against commercial oils and standard antibiotics the *in vitro* activity was modest. All the Myrtaceous oils (*L. scoparium, K. ericoides, M. alternifolia, M. leucadendron, M. viridiflora, M. quinquenervia*) were active against all the bacteria except *P. aeuroginosa*; while oil from the wood of *C. intratropica* belonging to the family Cupressaceae, exhibited little or no inhibition of the growth of all the bacteria tested.

L. scoparium was the most active against Gram positive organisms among the tested oils a result that was in concordance with the finding of Harkenthal *et al.*, (1999). The oil showed high selectivity against *S. epidermis*, exhibiting relatively a high inhibition zone (16 mm). On the contrary, the efficacy of *Melaleuca* oils was non-selective as the inhibition zones showed little variation between each other (ZI was 2 mm for *M. quinquenervia* and 3 mm for *M. leucandron*). The essential oil of *K. ericoides* was active against Gram positive organisms while it had little activity against Gram negative organisms.

As expected ciprofloxacin, a broad spectrum antibiotic, had the highest efficacy against *E. coli, B. cereus*, and *B. subtilis* (Australian Medicine Handbook, 2008). Gentamicin, an amino glycoside antibiotic, was the only antibiotic which exhibited some activity against Gram negative *P. aeuroginosa.* Penicillin-G was almost ineffective towards tested Gram positive organisms.

4.8.2.2 Antifungal activity

Antifungal activity of *Callitris* spp. oils was compared against 7 Australian essential oils and 3 pharmaceutical antifungal products, with a view to identifying those

essential oils which may have potential for the development as therapeutic products or may be a cheaper replacement for already available products. The positive controls chosen were those commercially available that are used in aromatherapy or for their purported antimicrobial activities. Table 4.4 presents the combined disc diffusion data of the essential oils and the commercial pharmaceutical products. The antifungal responses varied with the compounds and pathogens used.

Among the tested *Callitris* spp. essential oils *C. rhomboidea* foliage oil exhibited activity against all the tested fungal organisms except *T. rubrum* downy strain. Inhibitory activities varied between the *Microsporum* strains (*M. canis* and *M. gypseum*). The most notable was the activity against *M. canis*, exhibiting a relatively high zone of inhibition (5.5 mm). Relatively moderate activity was exhibited against *Ph. infestans*.

It was of particular interest that the *C. rhomboidea* leaf oils inhibited the growth of *M. canis*. It relatively inhibited 5x ZI in comparison with the tree oil and inhibition was 2x ZI in comparison with commercial proprietary antifungal agents at the tested concentrations (Fig 4.1 and Fig 4.2). None of the *Callitris* oils has been reported to be active against *M. Canis* infections.

Table 4.4 Zone of inhibition (ZI) as excess radius from the susceptibility disc for essential oils from different organs of *Callitris* spp., commercial oils and proprietary pharmaceutical products versus a range of fungi.

Sample	*E floccosum*		*M canis*		*T rubrum* granular		*Ph infestans*		*T.mentagrophyte* var interdigitale		*T rubrum* downy strain		*C albicans* (ATCC 6505)		*M* gypseum		*T. soudanese*	
	ZI (mm)		ZI (mm)		ZI (mm)		ZI (mm)		ZI (mm)		ZI (mm)		ZI (mm)		ZI (mm)		ZI (mm)	
	3 days	7 days	3 days	7 days	3 days	7 days	3 days	7 days	3 days	7 days	3 days	7 days	1 day	2 days	3 days	7 days	3 days	7 days
C oblonga leaf oil	1 0	1 0	0 0	0 0	3 5	3 5	1 0	2 0	1 5	2 0	5 5	5 5	2 0	5 0	0 0	0 0	0 0	0 0
C rhomboidea leaf oil	0 0	1 0	5 5	5 5	5 0	5 3	3 5	4 0	5 0	6 5	0 0	0 0	4 0	5 0	2 5	3 0	4 0	4 0
C. rhomboidea root oil	0 0	0 0	0 0	0 0	0 0	0 0	1 0	1 0	0 0	0 0	1 0	1 0	1.0	1 0	0 0	0 0	0.0	0 0
C. oblonga root oil	0 0	0 5	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	6 0	6 0	0 0	0 0	0 0	0 0
M. alternifolia	2 0	2.0	1.0	1 5	3 0	3 3	1 0	1.2	6 0	7 0	1 5	1 5	7.0	11 0	2 0	2 0	2 0	3 0
K. ericoides	0 5	1 0	1 0	1 0	3 0	4 0	2 0	2 3	2 0	2 5	2.5	2 5	5.0	7.5	2.0	2.0	3.5	4 0
L scoparium	2 0	2 0	3 0	4 0	4 3	6 0	2 5	2 5	2 0	2 5	2 0	2 8	1 0	1.5	1 0	1 0	2 0	2 0
M. leucadendron	2.0	2.0	1 0	1.5	4 5	5.3	3 0	4 3	3 0	3 5	3 5	4.0	1.0	1 5	3 5	4 0	4 5	5.0
M Viridiflora	2.0	2 0	1.0	0.5	2 9	3 3	2 0	2 5	9 0	10 0	1 5	2 1	10 0	12 0	2 0	3 0	4 0	4 0
M quinquenervia	1 0	1 0	1.0	1 0	3 8	4 0	2.0	2 0	5 0	5 0	1 5	2 0	5 0	8 0	1 0	1 0	5 0	5 0
C intratropica	0 0	0 0	0 0	0 0	0 0	0 0	1.0	1 0	1 0	1 5	0 0	0 0	ND[a]	ND	ND	ND	ND	ND
Lamisil	5 0	6 0	0 0	0.0	2 0	4 0	3 0	3 0	4 0	4 0	2 0	3 8	3 0	5 0	5 0	5 0	4.0	4 0
Nilstat	2.5	2.5	2 0	0 5	2 0	2 6	0 0	0 0	1 0	1.0	0 0	0 0	1 0	1 8	1.0	1.0	1.0	1.0
Nizoral	7.0	9.0	1 0	1 0	1 5	1 6	0 0	0 0	1 0	3 0	5 1	7 0	6 0	10 0	9 0	10 0	10 0	10 5

[a]Not determined

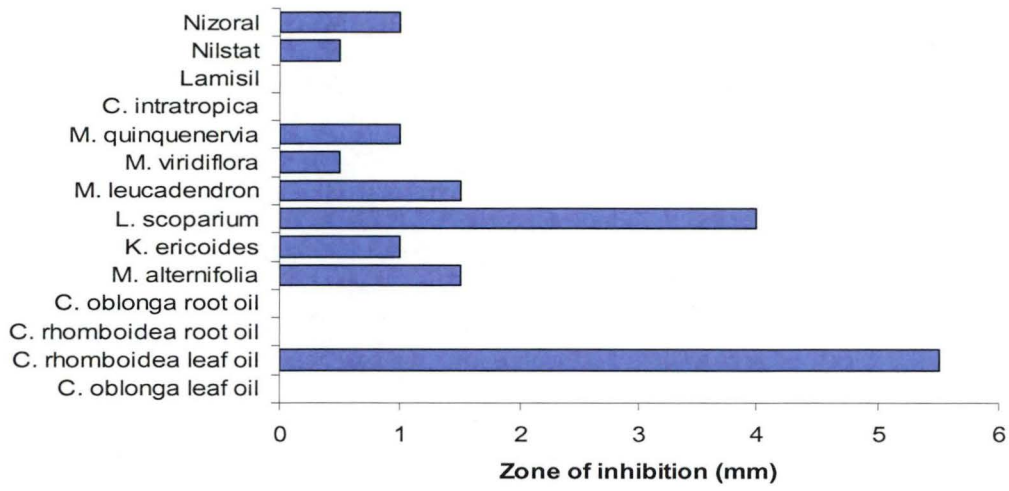


Figure 4.1 Comparisons of susceptibilities of essential oils and proprietary pharmaceutical antifungal agents against *M. canis* expressed in ZI (mm) as excess radius from the susceptibility disc.

By contrast *C. oblonga* foliage oil exhibited relatively high activity against *T. rubrum* granular strain; moderate activity against *T. rubrum* downy strain and *C. albicans*; and least activity against *E. floccosum* and *T. mentagrophytes* var interdigitale.

Root oil novel to the Cupressaceae family, displayed meagre activity against the tested fungi. *C. rhomboidea* root oil exhibited some activity against *Ph. infestans*, *T. rubrum* downy strain and *C. albicans*, while *C. oblonga* exhibited activity only against *C. albicans*. These results, considering the wide variety of fungi screened, it appear to disprove our hypothesis that root oil may have protective antifungal or antimicrobial activities.

Myrtaceous oils tested exhibited a varying degree of inhibition against dermatophytes. Among *Trichophyton* spp. strains, manuka oil (*L. scoparium*) exhibited the highest activity against *T. rubrum* granular strain while niaouli oil (*M. viridiflora*) exhibited the highest activity against *T. mentagrophyte* var interdigitale. Myrtaceous oils

exhibited a moderate activity against *T. rubrum downy* strain and *T. soudanese*. Myrtaceous oils exhibited moderate activities against *Microsporum* spp., and *Epidermophyton* spp. Tea tree oil and niaouli oils exhibited potent activity against *C. albicans* while manuka and cajuput oil revealed the least activity. None of the myrtaceous oils exhibited potent activity against the plant fungus *Ph. infestans*, which causes serious potato disease known as late blight disease.

In general among the proprietary antifungal agents Nizoral shampoo containing 0.2% ketoconazole exhibited the highest activity against all the tested dermatophytes with the only exception being *T. rubrum* granular strain which was inhibited more by Lamisil containing 1% terbinafine hydrochloride.

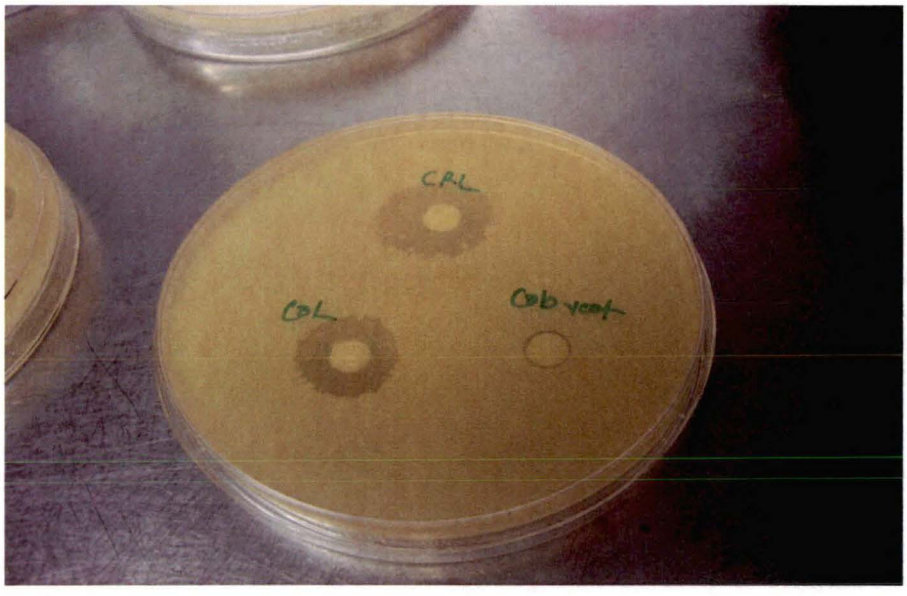


Figure 4.2 Plates incubated with *T. rubrum* granular strain showing activity as zone of inhibition (mm) as excess radius from the susceptibility disc containing *Callitris* oils.

M. canis infection is endemic in many show catteries in the United States and Europe, as evidenced by the isolation of 35 per cent of animals sampled as cat shows (Deboer and Moriello, 1994). Infected stray cats could be a reservoir for human infections.

As described in Chapter 2, the major compounds reported from the *C. rhomboidea* leaf oil were α-pinene, acetates (geranyl acetate, citronellyl acetate, and neryl acetate) and monoterpene alcohols (citronellol, geraniol and nerol). Investigation by Hammer *et al.*, (2003) has shown that the presence of the alcohol moiety is a major determinant of antifungal activity of tea tree oil constituents (*M. alternifolia*). The activity of the monoterpene alcohols has been attributed to their interaction with cellular membranes (Sikkema *et al.*, 1995). At relatively low concentration, these interactions may result in changes such as inhibition of respiration or alteration in permeability and at higher concentration effects such as total loss of homeostasis, gross membrane damage and death may occur (Hammer *et al.*, 2003).

Various compounds, including alcohols, aldehydes, fatty acid derivatives, terpenoids and phenolics, exist in plant essential oils. Jointly or independently, they contribute to the antifungal activities (Meepagala *et al.*, 2003). Analysis done by Yousef *et al.*, in 1978, demonstrated that geranyl acetate had no activity against dermatophytes. There are no reports on the antifungal activity of citronellyl and neryl acetate.

Citronellol and geraniol have been reported to be active against dermatophytes such as *Trichophyton* spp. (Shin and Lim, 2004). Lee *et al.*, (2008) demonstrated potent (100%) activity of citronellol and geraniol against the plant fungus *Phytophthora cactorum* at a concentration of 0.028 mg/ml air concentration. As tabulated in Table 2.2, geraniol and citronellol were present in *C. rhomboidea* leaf oil while only citronellol was present in *C. oblonga* leaf oil. This could possibly explain the activity of the *Callitris* leaf oils against *Ph. infestans*. The higher zone of inhibition of *C. rhomboidea* oil against *Ph. infestans* could be due to the synergistic activity of geraniol and citronellol.

Evaluation of activity of geraniol against dermatophytes done by Rao *et al.*, 2005, concluded that it possessed significant (>50%) activity against *T. rubrum*, *T. mentagrophytes*, *M. gypseum*, and *E. floccosum* at 0.5µl/ml concentrations. Furthermore when geraniol was incorporated into a formulation containing essential oil of *M. spicata*, the dermatophytic effects of the formulation along with shelf life were increased (Khanuja *et al.*, 2005).

4.8.2.3 Isolation of antifungal constituents

4.8.2.3.1 Fractionation of oil

Table 4.5 shows the activity of the fractions of *C. rhomboidea* leaf oil against *M. canis*, as was analysed by disc diffusion. Table 4.6 shows the GC-MS results of the analysis of *C. rhomboidea* leaf oil fractions.

Table 4.5 Zone of inhibition (ZI) as excess radius from the susceptibility disc of *C. rhomboidea* leaf oil and its fractions against *M. Canis*

Compounds	Zone of inhibition (mm)
C. rhomboidea leaf oil	5.5
C. rhomboidea fraction 1	0.5
C. rhomboidea fraction 2	0.0
C. rhomboidea fraction 3	0.0
C. rhomboidea fraction 4	2.5
C. rhomboidea fraction 5	6.5
C. rhomboidea fraction 6	6.5
C. rhomboidea fraction 7	6.5
C. rhomboidea fraction 8	6 0
C. rhomboidea fraction 9	5.5
C. rhomboidea fraction 10	5.5
C. rhomboidea fraction 11	5.0
C. rhomboidea fraction 12	4.5
C. rhomboidea fraction 13	4.5
C. rhomboidea fraction 14	4.5

As expected from our previous disc diffusion tests *C. rhomboidea* leaf essential oil and its fractions exhibited significantly potent inhibitory activity against *M. canis*. Among the tested samples, fraction numbers 6 and 7 exhibited the most potent activity. Fractions 1, 2 and 3 were rich in acetates (Table 4.6) and did not exhibit any significant activity against *M. canis*. Therefore it could be assumed that geranyl, neryl and citronellyl acetates that formed the major part of the leaf oil, were not active against *M. canis*. Fig 4.3 shows the percentage of total ion current determined by GC-MS of acetates and alcohols from different fractions of *C. rhomboidea* leaf oil and Fig 4.4 shows the zone of inhibition (mm) exhibited by different fractions against *M. canis*.

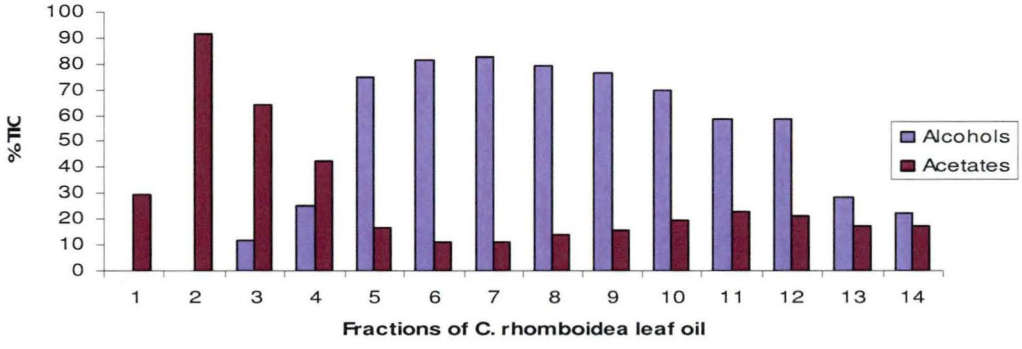


Figure 4.3 Percentage of total ion current of acetates and alcohols from different fractions of *C. rhomboidea* leaf oil.

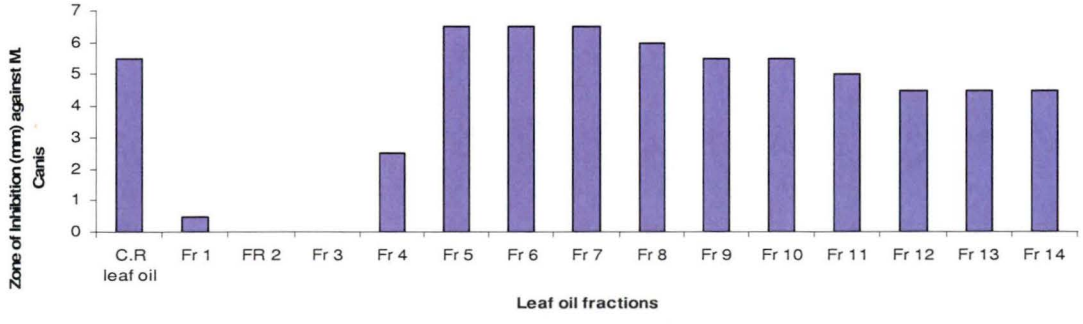


Figure 4.4 Comparisons of susceptibilities of C. rhomboidea leaf essential oil and its fractions against *M. canis* expressed in ZI (mm) as excess radius from the susceptibility disc.

Table 4.6 Composition of *Callitris rhomboidea* leaf essential oil and its fractions computed from the GC peak areas as determined by GC-MS. Peaks are listed in elution order on the VF5-ms column.

Compound	KI	*C. rhomboidea* leaf oil	*C. rhomboidea* leaf oil fractions													
			Fr* 1	Fr 2	Fr 3	Fr 4	Fr 5	Fr 6	Fr 7	Fr 8	Fr 9	Fr 10	Fr 11	Fr 12	Fr 13	Fr 14
α-Pinene	935	42.2	9.8													
β-Pinene	974	1.1														
Myrcene	988	1.3	2.9													
δ-3-Carene	1009		5.7													
Limonene	1032	1.0	4.7													
Terpinolene	1088		1.1													
Linalool	1097				7.1	11.4	8.6	4 2	2.7	2 2	1.8	1.4	1.3	1.5		
Citronellal	1150	1.1	1.0							1.1						
Isopulegol	1153				1.4	4.6	3.9	1.8	1.2					1.1		
Camphor	1154			1.9	4.1											
α-Terpineol	1200						3.1	4 0	3.8	3.5	3.1	2.5	2.8	1 4		
Citronellol (+some nerol)	1226	5.3			1.2	9.6	25.8	27.1	24.2	21.9	20.8	19.5	16.6	17.4	9.6	7.6
Geraniol	1253	6.3				2.6	22 7	40 7	48.4	48.1	47.7	42.4	33.9	34.1	17.6	13 9
Piperitone	1259							1.0	1.1	1.0	1 1					
Methyl citronellate	1262	1.1					1 1									
Bornyl acetate	1289		1.1	1.8	1.1											
Citronellic acid	1317												7.2	7.7	4 4	

Table 4 6 continued...

Compound	KI	*C. rhomboidea* leaf oil	*C. rhomboidea* leaf oil fractions													
			Fr* 1	Fr 2	Fr 3	Fr 4	Fr 5	Fr 6	Fr 7	Fr 8	Fr 9	Fr 10	Fr 11	Fr 12	Fr 13	Fr 14
Carveyl acetate	1336	1.4														
Citronellyl acetate	1349	6.7	11.1	17.7	11.1	7.5	2.9	2.2	2 2	2.8	3.2	4.1	5 1	4 7		
Neryl acetate	1358	5.5	10 1	23 7	16.3	11.7	4 3	3.0	2.9	3 5	3.9	4.9	5 8	5 4		
Geranyl acetate	1378	11 8	16.3	47.3	36.7	26.0	9 4	6.3	6 3	7 5	8.7	10.6	12 2	11.1		
Thujopsene	1444	2.2	8 1													
Unknown compound 7	1461	3.5	11.5													
Bicyclogermacrene	1502		1.2													
Spathulenol	1588	1.0			3.7	5 5	3 1	1.4	1.0	1 1	1.0	1 3	1 3	1.3		
Widdrol	1619				1 1	2.1	1.7		1.1							
Farnesol isomer	1719				2 3	6.3	6.1	2 4		1.3	1 2	1.7	1.9	1.9	1.1	1.0
Farnesyl acetate	1834		1.0	1 2												
Artefacts (Hydrocarbons)					2.2	1 1					1 0	3.2	3 7	4.3	56.8	69 2
Total acetates			29 5	91.6	64.0	42.6	16 6	11.4	11.3	13 9	15.8	19 3	23.1	21.2	17.4	17 2
Total alcohols					11 9	25.1	74 6	81.6	82.6	79 1	76.6	69.7	58 6	58.4	28.3	22.4
Total		91.5	85.6	93.6	88 3	88.5	88.4	94.1	93.8	94 0	94.0	90.3	91.8	92.0	90.0	91.6

* Fractions

The inhibitory activity expressed as ZI, of the fractions exhibited linearity with the alcohol fractions of the oil. Thereby, it was concluded that alcoholic compounds could be the responsible compounds for the inhibitory activity of the oil against *M. canis*. The alcohol fractions were chosen for MIC and MFC studies.

4.8.2.4 Minimum inhibitory concentration

The main alcohol components (Geraniol, nerol, and citronellol) of the *C. rhomboidea* leaf oil were purchased as pure (≥99%) compounds. The MIC and MFC results are tabulated in Table 4.7.

Table 4.7 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *C. rhomboidea* leaf oil in comparison with geraniol, nerol and citronellol against *M. canis*.

Bioassay	*Microsporum Canis*			
	C rhomboidea leaf oil Vol (%)	Geraniol Vol (%)	Nerol Vol (%)	Citronellol Vol (%)
MIC	0.25-0 13	0.13-0.06	>8.0	2 0-1 0
MFC	1.0	1.0	>8.0	>8 0

The MIC value of the pure geraniol (0.13-0.06 vol %) against *M. canis* was lower than the *C. rhomboidea* leaf oil (0.25-0.13 vol %). Although the MFC values of the citronellol was greater than 8% v/v, the citronellol inhibited the growth of the fungi (MIC) at a concentration of 2.0 -1.0% The results of this study demonstrate that the antifungal activity of the *C. rhomboidea* leaf oil could be due to the presence of geraniol and citronellol in the sample. This implies that the *C. rhomboidea* oil could be effective in the treatment of infections caused by *M. canis*.

Based on our disc diffusion studies and MIC results, the relatively stable monoterpene primary alcohols, citronellol and geraniol, exhibited inhibition of *M. canis*. Terpene alcohols could be clinically practical antifungal agents because of their stability,

especially at different pH conditions, while the clinical utility of phenolic compounds such as eugenol or thymol may be restricted at higher pH conditions (Shin and Lim, 2004).

It is interesting that *C. rhomboidea* oil was most potent against *M. canis* among all the tested dermatophytes. Unfortunately the antifungal mechanisms of action of the essential oils have not been clarified. The inhibitory mechanism of the terpenes against fungi was considered to be associated with fungal cell wall destruction, as was the case with ketoconazole, an antifungal drug (Shin and Lim, 2004).

M. canis is a filamentous fungus which acts on dermatophytes in humans and cattle by digesting keratin. *M. canis* infections represent a major public health problem in many European countries and Israel (Maraki and Tselentis, 2000). In a study conducted by Maraki and Tselentis (2000), on the epidemiology of *M. canis* infections in Crete, Greece, over a 5 year period it was found that *M. canis* constituted 24.5% of all the dermatophytes isolated during the study period. It was also observed that women were more frequently affected than men and cats were the commonest source of infection.

Therapeutic use of essential oils may provide an alternative solution for the rapid development of fungal resistance and drug-drug interactions that are problematic with the currently available common antifungal therapeutics. Further *in vivo* experiments are necessary to assess the potential for therapeutic application of *C. rhomboidea* leaf essential oil against *M. canis*.

4.8.3 Antimicrobial and antifungal activities of *Callitris* spp. extracts

In this study, the extracts of root, leaf, stem, bark and fruits of two *Callitris* spp. (*C. rhomboidea* and *C. oblonga*) were investigated for their antifungal activities against two dermatophytes (*T. mentagrophytes*, *M. canis*), an isolate of the yeast-like fungus (*C. albicans*) and antimicrobial activities against Gram negative *Escherichia coli* and Gram positive *Staphylococcus aureus*. The results of disc diffusion are tabulated in Table 4.9.

Results obtained from the controls indicated that the solvent had no effect on the growth of microorganisms and the organisms grew well during incubation. Among the extracts studied none of the extracts exhibited strong activity against Gram positive *S. aureus*. By contrast *C. oblonga* fruit methanolic/petroleum spirit and *C. oblonga* leaf petroleum sprit extracts exhibited the highest activity against the Gram negative bacterium *E. coli*.

C. oblonga fruit methanolic fraction and *C. rhomboidea* leaf methanolic fraction exhibited a relatively high inhibition zone diameter (7 mm) against *M. canis*; while *C. oblonga* bark methanolic extract was particularly active against *T. mentagrophytes* and *C. albicans*. In general the polar methanolic fraction exhibited a relatively higher inhibitory effect than the respective petroleum spirit extracts, except *C. rhomboidea* root petroleum ether extract, against the tested fungi.

Table 4.8 Zone of inhibition (ZI) as excess radius from the susceptibility disc of *Callitris* spp. extracts against *a* range of microorganisms.

Samples	Mass applied per disc (mg)	*E coli*	*C. albicans*	*S. aureus*	*T. mentagrophytes*	*M canis*
		ZI (mm)	ZI	ZI	ZI	ZI
C. rhomboidea root methanolic extract	10	0 0	10 0	1 0	1 0	1.0
C. rhomboidea leaf petroleum extract	10	0 0	1.0	1.0	1 0	3 5
C. rhomboidea leaf methanolic extract	10	0 0	1.0	0 5	2.0	7 0
C. rhomboidea stem petroleum spirit extract	10	0.0	7.0	0 0	1.0	0 5
C. rhomboidea stem methanolic extract	10	0 0	7 5	0 5	3.0	1.0
C. rhomboidea bark petroleum spirit extract	10	0.0	0 0	1.0	1.0	1 5
C oblonga root petroleum spirit extract	10	0.0	2 0	1.5	6 0	6 0
C. oblonga leaf petroleum spirit extract	10	4 0	0 0	2.0	2.0	2.0
C. oblonga leaf methanolic extract	10	0 0	4.5	2.0	6.5	6 5
C. oblonga bark petroleum spirit extract	10	0.0	0 5	1.0	0.5	0.0
C. oblonga bark methanolic extract	10	1.5	12 0	1 0	7.0	1.5
C. oblonga stem petroleum spirit extract	10	0.0	1 5	1 0	1 0	1.0
C. oblonga stem methanolic extract	10	6.5	2.5	1 0	6.0	6.0
C. oblonga fruit petroleum spirit extract	10	5.0	0.5	1.0	1 0	2.0
C. oblonga fruit methanolic extract	10	0.0	2.5	1.0	7 0	7 0
Petroleum spirit balnk	100	0 0	0.0	0 0	0.0	0.0
Methanol blank	100	0 0	0 0	0.0	0.0	0 0

By contrast to SD root oil, *C. oblonga* root petroleum ether extracts exhibited relatively high inhibitory activity against *T. mentagrophytes* and *M. canis*. In all the above extracts there was a high proportion of diterpenoids which is common in coniferous trees (Cox *et al.*, 2007). It could be the possible explanation for the high inhibitory activity of the root oils against fungi. The nature and the proportion of individual constituents of an essential oil/extracts and their synergistic effect on antimicrobial activity of essential oil have been reported by Pattnaik and co-workers (1997).

4.9 CONCLUSION

Steam distilled root oils of *Callitris* spp. exhibited low antifungal activity, while extracts from the root exhibited some degree of antifungal activity. *C. rhomboidea* leaf oil has some potentially useful activity against *M. canis*, better than the commercial antifungal agents at the tested concentrations. Activity against *M. canis* was shown to be primarily due to the presence of geraniol and citronellol in the leaf oil.

CHAPTER 5

Antioxidant Assays, Allelopathic Effects and Physico-Chemical Properties of the Oil and Extracts of *Callitris* spp.

5.1 ANTIOXIDANT ASSAY

5.1.1 Introduction

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effect of free radicals in the human body, and to prevent the deterioration of fats and other constituents of foodstuffs. The presence of free radicals on biological system was discovered around 50 years ago (Commoner *et al.*, 1954). In both applications there is a preference for antioxidants from a natural source rather than from synthetic sources (Abdalla and Roozen, 1998). Plant antioxidants are composed of a broad variety of different substances like ascorbic acid and tocopherols, polyphenolic compounds, and terpenoids (Grassmann, 2005).

Free radicals are atoms or ions containing unpaired electrons that cause damage to our cells. They harm our immune system leading to many degenerative diseases. Free radicals are formed by our cells being exposed to a variety of environmental factors such as radiation, chemicals, pollution, smoke, drugs, alcohol, pesticides and sunlight. Free radicals are also produced via various cellular processes such as when our bodies utilise stored fat for energy.

Free radicals are recognised to play twin roles in biological systems. They can either be beneficial or harmful to living systems (Li *et al.*, 2007). High concentration of free radicals can induce the damage of cellular structures, including lipids and membranes,

proteins and nucleic acids, which leads to several diseases such as cancer, atherosclerosis (?) and cardiovascular diseases, inflammatory lung diseases, immune dysfunctions, and neurodegenerative disorders (Li *et al.*, 2007). At moderate concentrations the free radicals have been discovered to play an important role as regulatory mediators in signalling processes. Thereby, they protect the cells from oxidative stress and re establish the redox homeostasis (Droge, 2002).

The major type of free radicals and their derivatives in living organisms are superoxide anion (O_2^-) and nitric oxide (NO). The superoxide ion is formed by the reduction of triplet-state molecular oxgen ($^3O_2$), this process being mediated by enzymes such as NAD(P)H oxidases and xanthine oxidases or non enzymatically by redox reactive compounds such as semi-ubiquinone compounds of the mitochondrial electron transport chain. The NO radical ($NO^{\cdot}$) is produced in higher organisms by the oxidation of one of the terminal guanido-nitrogen atoms of L-arginine (Droge, 2002).

Antioxidants work by donating an electron to free radicals to convert them to harmless molecules. This protects cells from oxidative damage that leads to aging and various diseases. Some antioxidant assays use the same mechanism to measure the efficacy of the test substance towards free radical elimination (Li *et al.*, 2007).

Essential oils are known to possess potential as natural agents for food preservation (Emami *et al.*, 2007). Essential oil components have been studied by Ruberto and Baratta (2000), and many of them have been classified as natural antioxidants and proposed as potential substitutes for synthetic antioxidants in specific sectors of food preservation. Phenols exhibited the highest antioxidant activity. Some monoterpene hydrocarbons, namely terpinolene, α- and γ-terpinene, showed a significant protective action, whereas oxygenated components, besides phenols and allylic alcohols,

manifested an appreciable activity. Sesquiterpene hydrocarbons and non-isoprenoid components showed a low antioxidant effect.

There are no published reports on the antioxidant activity of the essential oils and extracts obtained from different parts of *C. rhomboidea* and *C. oblonga* plants.

There are different antioxidant assays available and, because the results rely on different mechanisms, they strictly depend on the oxidant/antioxidant models employed and on the hydrophyllic/lipophyllic balance (Emami *et al.*, 2007).

In this study, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities of the essential oils and methanolic extracts obtained from different parts of the plant were studied. The molecule of DPPH is characterised as a stable free radical by virtue of the delocalisation of the unpaired electron over the molecule as a whole. The delocalisation also gives rise to the deep violet colour, characterised by the absorption band in methanol solution centred at about 490 nm. As the DPPH is mixed a substance that can donate an electron then this gives rise to the reduced form with the loss of the violet colour (Molyneux, 2004).

5.1.2 Materials

5.1.2.1 Chemicals

The chemicals used for the experiment were gallic acid (Sigma-Aldrich, Sydney, Australia); methanol AR (BDH/Merck, Melbourne, Australia); 2,2-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, Sydney, Australia) and microtitre plates from Iwaki (Tokyo, Japan).

5.1.2.2 Instruments

A microtitre plate reader Model 680 from Bio-Rad (Sydney, Australia) was used to measure the absorbance of the sample.

5.1.2.3 Essential oils

Leaf and root steam distilled oils as well as solvent extracts from different organs of *C. rhomboidea* and *C. oblonga* plants were obtained as is described in Chapter 2.

5.1.3 DPPH radical scavenging assay method

The antioxidant assay was performed as is described by Jirovetz *et al.*, 2006. DPPH was prepared by dissolving approximately 5 mg of DPPH in 100 ml of methanol. The absorbance of 250 µl of this solution in a microtitre plate was measured and the concentration adjusted with methanol to give an initial absorbance of 1.3 at 490 nm. The solution was kept in the dark after preparation. Gallic acid standards were prepared in the following concentrations: 200, 100, 50, 25, 12.5, 6.2, and 3.1 µg/ml with methanol.

C. rhomboidea and *C. oblonga* leaf and root oils were prepared as 10% v/v solutions in methanol and diluted 5- and 25-fold with methanol. Plant solvent extracts of *Callitris* species were prepared by dilution to 100, 20, and 4 mg/ml in methanol.

Aliquots (12 µl) of different concentrations of standard and control (methanol) were pippetted into wells of a microtitre plate in triplicate. Aliquots (12 µl) of plant extracts and essential oil solutions were pipetted into the wells of the microtitre plate in triplicate. Two hundred and fifty microlitres of DPPH solution were rapidly pipetted into each well. The microtitre plate was incubated in the dark for 30 minutes and read at 520 nm.

Absorbance versus concentration calibration curves were constructed for the standard gallic acid (Fig 5.1). The maximum absorbance value was taken as the average of three control determinations (no antioxidant added). The maximum absorbance value was 1.24 absorbance units (AU). The minimum absorbance value was taken as the average of three determinations containing excess antioxidant. The minimum absorbance was 0.17 AU. From the standard curve the antioxidant activity of the samples was read.

5.1.4 Results and discussion

Calibration curve of the gallic acid and the absorbance are shown in Fig 5.1.

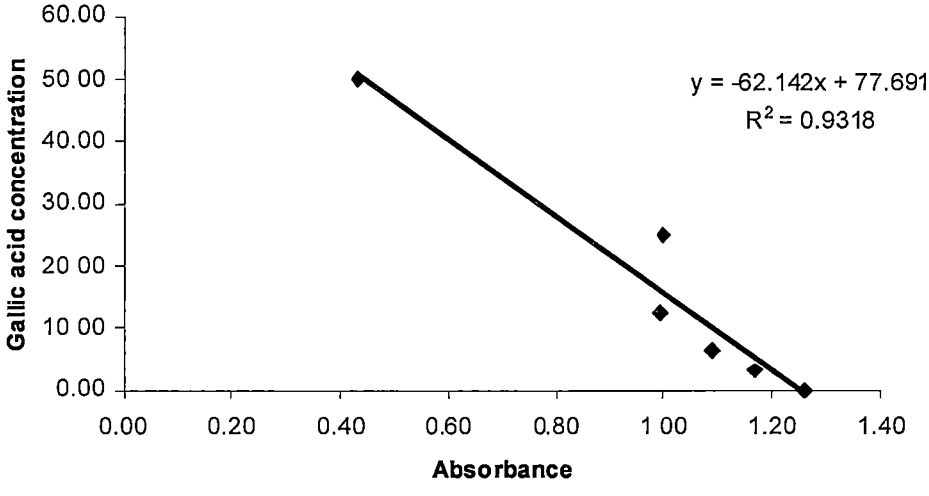


Figure 5.1 Calibration curve of gallic acid (μg) and absorbance (AU) in the DPPH antioxidant assay.

In this assay the, abilities of the test compounds (both the essential oil and the extracts) to donate hydrogen atoms or electrons were measured spectrometrically. The hydrogen atoms or electron donating abilities of the essential oils and corresponding extracts were from the bleaching of purple coloured methanol solution of DPPH. Table 5.1 summarises the activity of each extract in the DPPH assay as its gallic acid equivalents (µg/ml).

Table 5.1 Antioxidant assay of the test compounds in the DPPH assay at different concentrations.

Essential oils and extracts	Gallic acid equivalents (μg/ml)		
	100%	25%	4%
C rhomboidea leaf oil	6 6	0 0	0 0
C rhomboidea root oil	15 6	6 2	0.0
C oblonga leaf oil	0.0	0.0	0 0
C. oblonga root oil	0.0	0.0	0.0
C. rhomboidea leaf methanolic extract	57.2	59.7	24.3
C. rhomboidea root methanolic extract	62 8	45.4	19.9
C. rhomboidea stem methanolic extract	59.1	41.2	31 0
C. oblonga leaf methanolic extract	63.4	60.9	26 1
C. oblonga fruit methanolic extract	55.9	40.1	34.3
C. oblonga bark methanolic extract	64.1	32.3	0.0
C oblonga stem methanolic extract	65.2	49 1	0.0

The tested *Callitris* essential oils showed very meagre antioxidant activity. Among the tested oils *C. rhomboidea* root oil possessed the highest antioxidant activity (15.6-6.2 μg/ml, gallic acid equivalents). The antioxidant activity of the essential oils is dependent on the composition of the oil.

According to Emami *et al.*, (2007) the major antioxidant compound in conifers was γ-terpinene. Very low antioxidant properties were exhibited by limonene, α-pinene and β-pinene. The antioxidant effect of leaf oils of *Juniperus communis* a conifer belonging to Cupressaceae family was analysed by DPPH assay and it was found to possess an antioxidant effect of 65% at a concentration of 4 μl/ml in comparison with the standard ascorbic acid (Emami *et al.*, 2007). The higher value was found to be partially due to the high amount of γ-terpene (7.2%) in the sample. How ever the fruit oil of *J. communis* was found to possess weak antioxidant activity (4%); partially due

to the low amounts of γ-terpinene (0.9%) and high amounts of α-pinene (33.3%) and β-pinene (20.8%), both of which were inactive in DPPH test.

As described in Chapter 2, the above leaf oils were rich in α-pinene (42.1%) and absence of γ-terpinene. It could be the possible reason behind the absence of antioxidant activity in the leaf oils. The low activity of the *C. oblonga* and *C. rhomboidea* root oils could possibly be due to the presence of a high percentage of sesquiterpene hydrocarbons and sesquiterpene lactones (Ruberto and Baratta 2000).

But the extracts exhibited high antioxidant activity. The methanolic extracts obtained from the stem of both *Callitris* spp. was the most potent among all the extracts studied. Investigations done by Lim *et al.*, 2002, found that methanolic extracts of the heartwood of *Juniperus chinensis* (Cupressaceae family) showed strong antioxidant activity. The major compounds isolated were flavanoids like quercetin, naringenin, taxifolin, aromadendrin and isoquercitrin. Therefore it could be inferred that polar compounds like flavanoids could be responsible for the antioxidant activity of the plant extracts. Although several flavanoids were identified from the *Callitris* family (Khan and Ansari, 1987; Gadek and Quinn, 1982; Ansari *et al.*, 1981; Khan *et al.*, 1979; Erdtman, 1955) the antioxidant potential of these compounds has not been reported.

Petroleum spirit extract and DCM extract was not studied for antioxidant activity since the water-insoluble part of the extract is insoluble in aqueous test media and its colour could have interfered with the spectroscopic measurements. Only the water soluble fraction of the extracts could be tested for their antioxidant effects.

The results of the present study suggest that the antioxidant activity of the essential oil depend on the composition of the oil. The solvent extracts possess higher antioxidant activity than the essential oils.

5.2 ALLELOPATHY

Allelopathy is commonly defined as any direct or indirect effect of one plant, including microorganisms, on another through the production of chemicals released into the environment (Takikawa, 2006). Chemicals are released into the environment in three different ways: exudation, leaching, and volatilisation (Parvez *et al.*, 2004). Phytotoxins and allelochemicals are released from either the above-ground (leaf, stem, bark, flower, fruits, seeds etc) or below ground (roots, tuber, etc) parts of the plant. These chemicals may accumulate and persist for a considerable time and thereby interfere with the growth of neighbouring plants. Plants use allelochemicals as 'biocommunicators' similar to pheromones in insects. These chemicals could also act in plant defence (Macias *et al.*, 2008).

The development of new bioactive molecules which could have potential application in pharmacology and agriculture has always been encouraged. Allelochemicals have been investigated with the main propose of finding a phytotoxic substance for use as a natural herbicide template. The role of plant allelochemicals towards plant defence could be modified as a potential advantage in weed control in agriculture. Analytical techniques and bioassays are the basic tools for the detection and understanding of allelopathy.

Many allelochemicals with different skeletal types have been described to date. They can be broadly classified as phenolic derivatives, including simple phenolics like benzoic acids, flavanoids, polyphenols, depsidones and other aromatic compounds of

lichen origin and terpenoids such as monoterpenoids, sesquiterpene lactones, quassinoids, benzoxazinoids and glucosinolates (Macias *et al.*, 2007).

Bioassays are defined as the assessment of the potency of a compound via its application-induced response to the subject (Govindarajulu, 1988). They are a necessary step for the isolation, purification and identification processes of allelopathic compounds (Veronneau *et al.*, 1997).

There is a generally held notion amongst the landholders whose properties and leases contain *Callitris* that the species litter has some detrimental effect on the growth of ground cover. The landholders view has been expressed in Harris *et al.*, (2003):

'....where pine trees are thick there is never any grass there- well pine trees, out of the leaves, they give out this poison and it poisons the ground for everything else so that only pine will grow in it. I don't know how right it is....but I've noticed that where there is a lot of pine trees there's not a blade of grass or anything...' (Interview with Wittenbri, Bugaldi, long time resident of Pillinga Forest, 17 November 1996)

This study explored the allelopathic effect of the *C. rhomboidea* and *C. oblonga* root and foliage oils on lettuce seeds. It was observed that these trees are remarkably free from any insect and disease attack and it was assumed that the oil obtained from the plant could have certain chemical properties for such resistance. We have observed that thickets of *C. rhomboidea* on the east coast of Tasmania have very few other plants growing in the leaf litter under them.

5.2.1 Lettuce seed bioassay

Numerous bioassays have been employed to analyse the activity of allelochemicals. One among them was lettuce seed (*Lactuca sativa*) bioassay. Lettuce seeds have been

used extensively as a test seed because of their easy availability, affordability, rapid germination and the production of repeatable and reliable results (Pennacchio *et al.*, 2005). The lettuce seed bioassay technique was employed in this study.

5.2.2 Materials

Lettuce seeds, agar from Oxoid (Adelaide, Australia), acetone from BDH (Melbourne, Australia), steam distilled oils (*C. rhomboidea* and *C. oblonga* plants), *Kunzea ambigua* oil from JJ Hood, Ducane Estate (Waterhouse, Tasmania) and tween 80 from Sigma Chemicals (Sydney, Australia) were used in this study.

5.2.3 Methods

The whole experiment schedule was designed as follows:

- Experiment 1; determine a suitable concentration of tween 80 that did not hinder plant growth.

- Experiment 2; determine the concentration-dependent effects of *Callitris* spp. leaf and root oils on the germination of lettuce seeds, negative control being agar medium alone, experiment duration being 7 days.

- Experiment 3; determine the effect of *Callitris* spp. oils on the growth of lettuce seeds for a prolonged period of time (21 days), positive control being *Kunzea ambigua* leaf oil and the negative control being acetone.

In every independent experiment, 10 seedlings were allowed to germinate and the root and shoot lengths were measured. Student's t-test was performed on all the tested samples and if the p value was less than 0.05, results were considered as significant. All the bioassays were done in duplicate.

5.2.3.1 Lettuce seed bioassay for determining the concentration of tween 80

One percent of agar in water was heated to 80 degrees for 20 minutes with stirring. Aliquots of agar medium (20 ml) were transferred into 50 ml glass beakers. The medium was allowed to cool and 20µl of tween 80 solution in water were added. tween 80 solutions were prepared in three different concentrations (0.001%, 0.01%, 0.1% v/v) in water. After the gel was formed 10 lettuce seeds were evenly spread over the surface of the agar. The beakers were kept in a place with ample sunlight. After 7 days the shoot and root length were measured.

5.2.3.2 Lettuce seed bioassay for essential oils

Agar was prepared as descibed in the section above and 20 ml aliquots were transferred into 50 ml beakers. The medium was allowed to cool and before setting tween 80 (0.001%) was added. Essential oil solutions of *Callitris* and *Kunzea* oil in acetone were pipetted into the medium at several concentrations beginning from the lowest concentration of 0.04 µl/20 ml to the highest concentration 20 µl in 20 ml in agar medium. The solutions were mixed thoroughly. After the gel had set 10 lettuce seeds were evenly spread over the entire surface. The beaker was covered with a watch glass and observed daily for growth. The length of the root and hypocotyl were measured after 7 days for Experiment 2 and 21 days for Experiment 3.

5.2.4 Results

5.2.4.1 Tween 80 experiment

Fig 5.2 shows the effect of tween 80 at three different concentrations on the growth of lettuce plants.

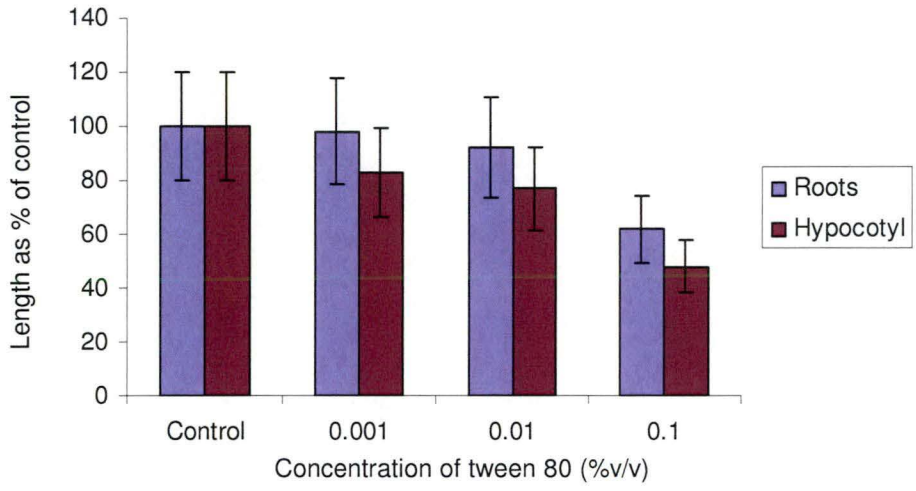


Figure 5.2 Effect of tween 80 at three different concentration (0.1, 0.01, 0.001 % v/v) on the growth of lettuce plants

The main drawback of the lettuce seed bioassay was the solubility of nonpolar substances. In order to increase the solubility of essential oils in agar solution tween 80 was added. Students t-test performed on the results from the growth test demonstrated that there was a significant inhibitory effect of 0.1% tween 80 on hypocotyl elongation. tween 80 appeared to have a greater effect on hypocotyl than on root growth. tween 80, at a concentration of 0.001% v/v was found not to interfere with the growth of the plants.

Polysorbate 80 (commercially also known as tween 80, a trademark of Croda International Plc, previously Uniqema/ICI) is a nonionic surfactant and emulsifier

derived from polyoxylated sorbitan and oleic acid. It was proposed as a dispersion agent for essential oils by several authors and it has been used to date in microbiological assays of essential oils for their MIC determination (Carson *et al.*, 1995).

This result is in accordance with Mendez *et al.*, 1967, which states that tween 80 has no direct effect in the elongation on *Avena coleoptile* section at a concentration of 0.1%. However tween 20 exhibited a toxic effect on plants at a concentration of 0.1% and the results obtained from tween 40 were erratic.

5.2.4.2 Activity of essential oils on lettuce seeds

Table 5.2 shows the effects of the essential oil from leaf and roots of *C. rhomboidea* and *C. oblonga* on the growth of lettuce plants shoot and root length.

As shown in Figs 5.3 and 5.4 the growth of root and shoot of lettuce seeds was drastically reduced in the three tested concentrations (20 µl, 4 µl, and 1 µl in 20 ml of agar medium) of *Callitris rhomboidea* oils. Students t-test performed on the *C. rhomboidea* root oils showed that as the sample concentration decreased to 0.2 µl/20 ml and 0.04 µl/20 ml, the inhibitory effect of the oil became insignificant. However in contrast *C. rhomboidea* leaf oil demonstrated inhibitory effects even at low concentrations such as 0.2 µl/20 ml and 0.04 µl/20 ml. In general the inhibitory effect was directly proportional to the concentration of the oil sample. Root and leaf oils of *C. rhomboidea* inhibited hypocotyl elongation more than root growth.

Students t-test performed with *C. oblonga* root and leaf oil samples demonstrated that inhibitory effect for 0.2 µl/20 ml and 0.4 µl/20 ml were insignificant (Figs 5.5 and 5.6). In general on comparison of the two *Callitris* species it was observed that *C.*

oblonga root oil possessed more inhibitory effect on root and shoot elongation of lettuce seeds than *C. rhomboidea* root oil; while leaf oil of *C. rhomboidea* was found to possess more inhibitory activity than the leaf oil of *C. oblonga* oil.

Table 5.2 Effect of *Callitris* spp. oils on the growth of the lettuce plants.

Sample	Concentration µl/20 ml	Non germinated seeds	Average root length (cm)	Standard deviation (roots)	Average hypocotyl length (cm)	Standard deviation (hypocotyl)
C. rhomboidea root oil	20.0	1	0.4	0.2	0.3	0.2
	4.0	2	0.6	0.5	0.5	0.3
	1.0	1	0.8	0.6	0.8	0.6
	0.2	2	1.0	0.6	1.4	1.0
	0.04	0	1.4	0.8	1.3	1.0
C. rhomboidea leaf oil	20.0	1	0.5	0.1	0.2	0.1
	4.0	1	0.6	0.4	0.4	0.2
	1.0	1	1 0	0.4	0.9	0.5
	0.2	0	1.3	0.8	1.1	0.8
	0.04	0	1.2	0.5	1.5	0.5
C. oblonga root oil	20.0	2	0.3	0.2	0.2	0.2
	4.0	0	0.4	0.2	0.3	0.2
	1.0	0	0.5	0.3	0.4	0.3
	0.2	1	1.1	0.4	1.0	0.4
	0.04	0	1.2	0.8	1.6	1.0
C. oblonga leaf oil	20.0	2	0.9	0.6	0.8	0.6
	4.0	0	0.9	0.4	0.9	0.5
	1.0	1	0.8	0.5	1.1	1.1
	0.2	0	1.2	0.7	1.8	1.3
	0.04	0	1.3	0.9	1.5	0.9
Blank sample	20.0	0	1.6	0.0	2.0	0.0

As is discussed in Chapter 2, the main constituents of the root oil from *C. rhomboidea* were longiborneol (23%), UC1 (6%), longifolene (5%), thujopsene (3%), α-chamigrene (2%), β-calacorene (2%), spathulenol (2%) and guaiol (1%); while the main constituents of the root oil from *C. oblonga* were columellarin (30%), UC19-30 were unidentified γ-lactones which accounted to 18%, longiborneol (4%), isosativene

(3%), and β-selinene (2%). None of the above said compounds are reported to possess allelopathic activity.

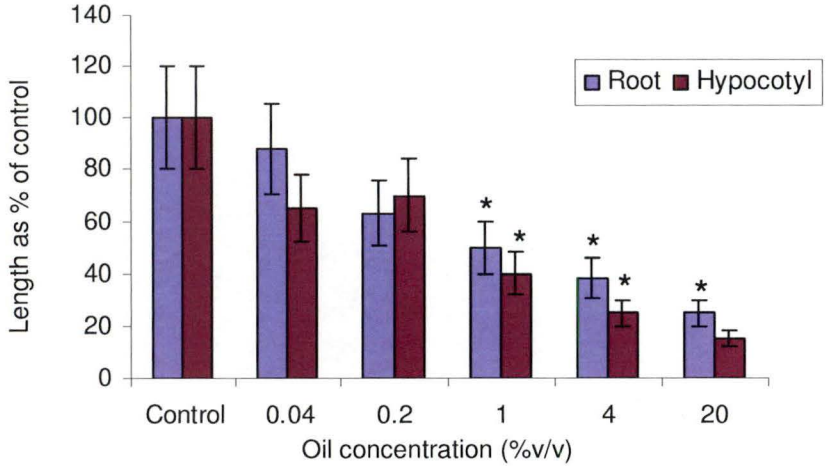


Figure 5.3 Effect of SD root oil (ml) of *C. rhomboidea* on the growth of lettuce plants compared to control (*p<0.05)

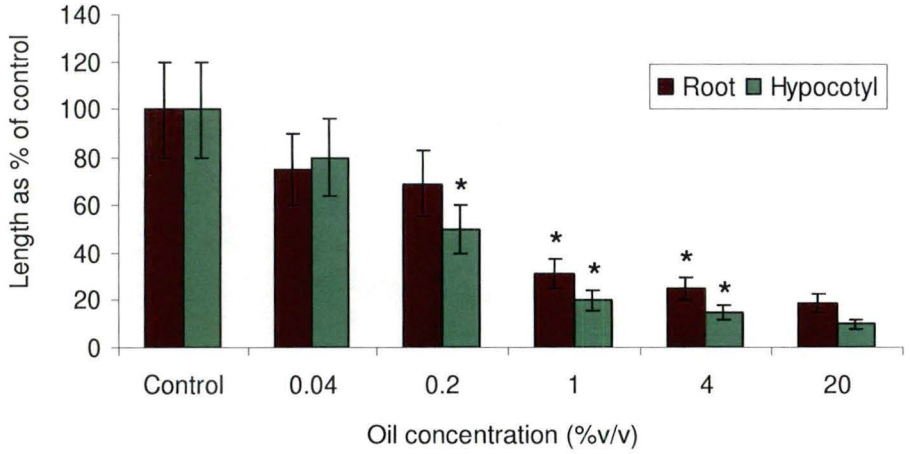


Figure 5.5 Effect of SD root oil concentration (ml) of *C. oblonga* on the growth of lettuce plants compared to control (*p<0.05)

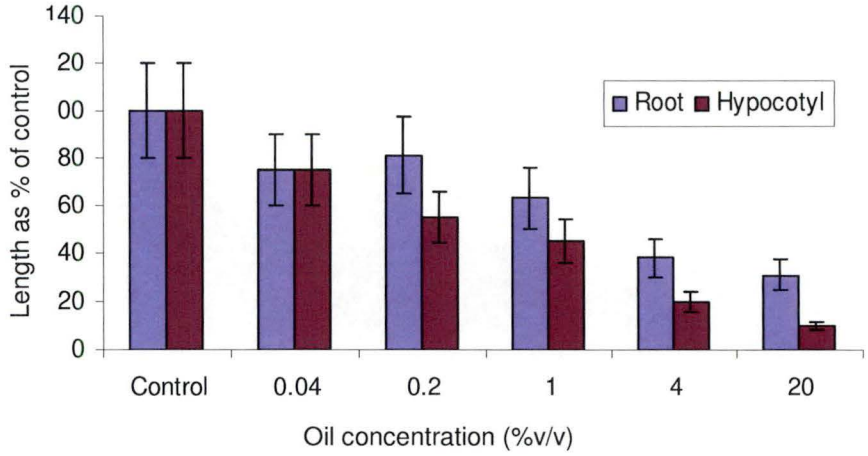


Figure 5.4 Effect of SD leaf oil (ml) of *C. rhomboidea* on the growth of lettuce plants compared to control

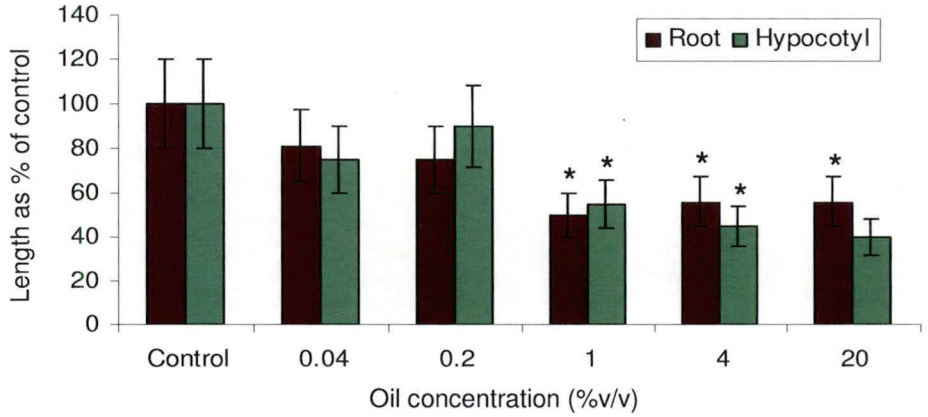


Figure 5.6 Effect of SD leaf oil concentration (ml) of *C. oblonga* on the growth of lettuce plants compared to control (*p<0.05)

Hoch *et al.*, (2000) demonstrated that the durability of timbers has a direct co relation with the allelopathic activity of the respective plant. Japanese cherry birch tree (*Betula grossa* var. *ulmifolia*) is famous for its good quality hardwoods and has strong resistance to birch leaf-miner (Hoch *et al.*, 2000). The resistance was due to the presence of salicylic acid. Similarly, *Callitris* timbers are known for their termite resistance and durable timbers. Durability of the timbers was reported to be due to the presence of columellarin (Watanabe *et al.*, 2005). Thereby, we are speculating that the inhibitory activity of the root oil and leaf oil of *C. oblonga* could be due to the presence of columellarin.

The leaf oil of *C. rhomboidea* mainly constituted α-pinene (42%), geranyl acetate (12%), neryl acetate (6%), and citronellyl acetate (7%); while the leaf oil of *C. oblonga* predominantly constituted α-pinene (42%), isopulegol (6%), columellarin (6%), citronellol (6%) and spathulenol (2%). None of the above mentioned compounds are investigated for their allelopathic effects. Therefore, it would be unwise to comment much further.

5.2.4.3 Activity of essential oils on lettuce seeds (long term)

Fig 5.7 and 5.8 shows the effect of *C. rhomboidea* root oil and *K. ambigua* oil on the growth of lettuce seeds.

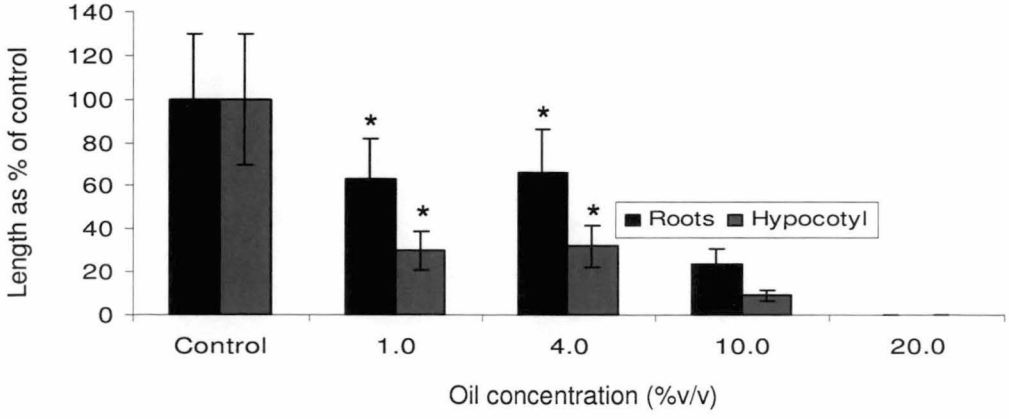


Figure 5.7 Effects of *C. rhomboidea* root oil on the growth of lettuce plants (*P<0.05)

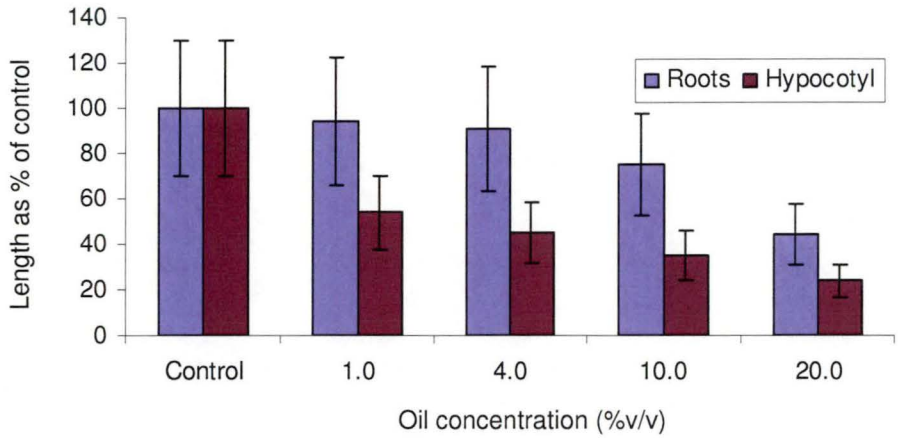


Figure 5.8 Effects of *K. ambigua* leaf oil on the growth of lettuce plants

The experiment clearly demonstrated that the *C. rhomboidea* root oil has an inhibitory activity on the lettuce plants. Students t-test performed on the root oil samples demonstrated that the inhibitory effect of the oil is significant. The highest concentration of root oil (20 µl in 20 ml) inhibited the growth of all lettuce plants. This led to the wilting of plants and their death (Table 5.3). It was also observed that the wilting of plants was directly proportional to the oil concentration.

Table 5.4 Effect of *C. rhomboidea* root oil and *K. ambigua* leaf oil in the wilting off and death of lettuce plants.

Sample	Concentration (µl/20 ml)	Wilted off lettuce plants
C. rhomboidea root oil	20	30
	10	17
	4	6
	1	4
K. ambigua leaf oil	20	9
	10	4
	4	0
	1	0
Blank sample		0

Control *K. ambigua* leaf oil sample also exhibited an inhibitory effect, but only at a higher concentration (20 µl/20 ml and 10 µl/20 ml). Statistical analysis exhibited that

as the concentration of the oil decreased below 4µl/20 ml the inhibitory effect of the oil was found to be insignificant. The kunzea oil sample did also induce wilting off (9 plants at 20µl/20ml), but it was far compared with the same concentration of *C. rhomboidea* root oil (30 plants at 20µl/20 ml).

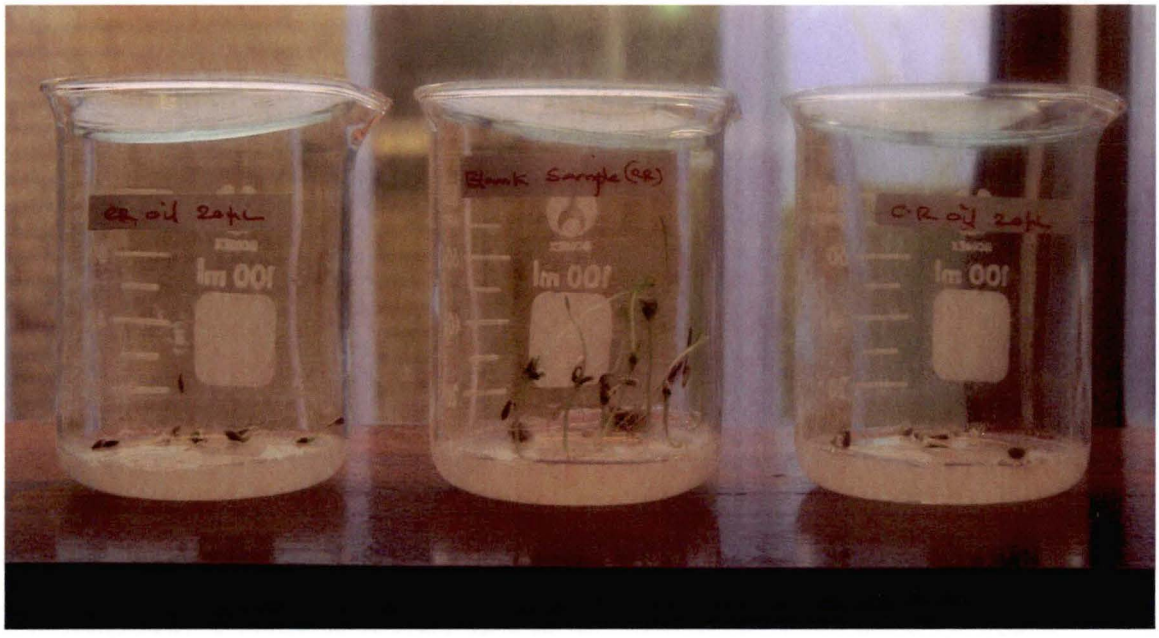


Figure 5.9 Comparison of growth inhibitory effects of *C. rhomboidea* oil (20 µl/20 ml) on lettuce seeds versus blank sample

5.3 PHYSICOCHEMICAL PROPERTIES

To prevent adulteration, physicochemical properties of essential oils should be determined. The physicochemical properties that were determined included boiling point (BP), optical activity, and refractive index (RI).

5.3.1 Materials and Instruments

The solvents used were acetone supplied by BDH (Sydney, Australia) and methanol AR supplied by BDH (Sydney, Australia). Microcapillaries for boiling point determination were prepared inhouse. Optical rotations were determined using a,

polarimeter model Autopol II, from Rudolph Research Analytical (Hackettstown, USA). Boiling points were determined using a melting point apparatus from Gallenkamp (Leicestershire, UK).

5.3.2 Method

5.3.2.1 Polarimetry

A 25 ml volumetric flask was rinsed with acetone, and dried using compressed air. Each essential oil (0.25 g) was weighed in a volumetric flask into which methanol was added to 25.0 ml. The sample cell of the polarimeter was filled with methanol without air bubbles and set to zero. After removing methanol from the sample cell, the essential oil solution was added to the sample cell. The angle of rotation (α) was recorded.

5.3.2.2 Boiling point determination

Boiling point was determined by using a microcapillary technique (Pavia *et al.*, 1990). The essential oil was loaded into a glass melting point tube having an inner diameter of 1 mm. A sealed capillary was suspended inside the melting point tube with an open end immersed in the oil sample. The melting point tube was placed into the melting point apparatus and heated until there was a steady stream of bubbles flowing from the open end of the microcapillary. Heat was turned off and sample was cooled. Eventually the bubbles stopped flowing and the essential oil was drawn up into the microcapillary. The temperature at which this occurred was recorded as the boiling point of the sample. The experiment was repeated three times with each essential oil and the mean values were recorded.

5.3.3 Results

Table 5.3 shows the boiling point and optical rotation values of *Callitris* oils.

Table 5.5 Boiling point and optical rotation values of *Callitris* oils

Essential oil	Boiling point (°C)	Optical rotation $^{20}[\alpha]_D$
C. rhomboidea root oil	140-148	+21 6°
C. rhomboidea leaf oil	135-142	+1.0°
C. oblonga root oil	135-140	+10 9°

The boiling point of a liquid is the corrected temperature at which the vapor pressure of the liquid reaches 101.3 kpa. The boiling point of root oils obtained from *Callitris* oils varied marginally. *C. oblonga* leaf oil was not analyzed due to the lack of the SD oil.

Optical rotation of a solution in the angle through which the plane of polarisation is rotated when polarised light passes through the solution (BP, 1988). Substances are designated as *dextro* or *laevo* rotatory according to whether the plane polarised light was rotated clockwise or anticlockwise. All the oil samples analyzed were *dextro* rotatory.

The specific rotation of a chemical compound (α) is defined as the observed angle of optical rotation α when plane-polarized light is passed through a sample with a path length of 1 decimeter and a sample concentration of one gram per one decilitre. The specific rotation of a pure material is an intrinsic property of that material at a given wavelength and temperature. The specific rotations of the samples were determined at 20 °C. The specific rotation of *C. rhomboidea* root oil was observed to be the highest

among the analysed samples. *C. oblonga* leaf oil was not analysed since there was not enough quantity of oil.

5.4 CONCLUSION

Results reported here could be considered as the first detailed report on the antioxidant, allelopathic and physicochemical properties of *C. oblonga* and *C. rhomboidea* essential oils and extracts. The antioxidant activities of the *Callitris* oils were relatively low. It has been demonstrated that the respective *Callitris* spp. essential oils possessed a strong inhibitory effect on plant growth. *Callitris* plant oils may be potentially useful as a template for the development of a herbicide.

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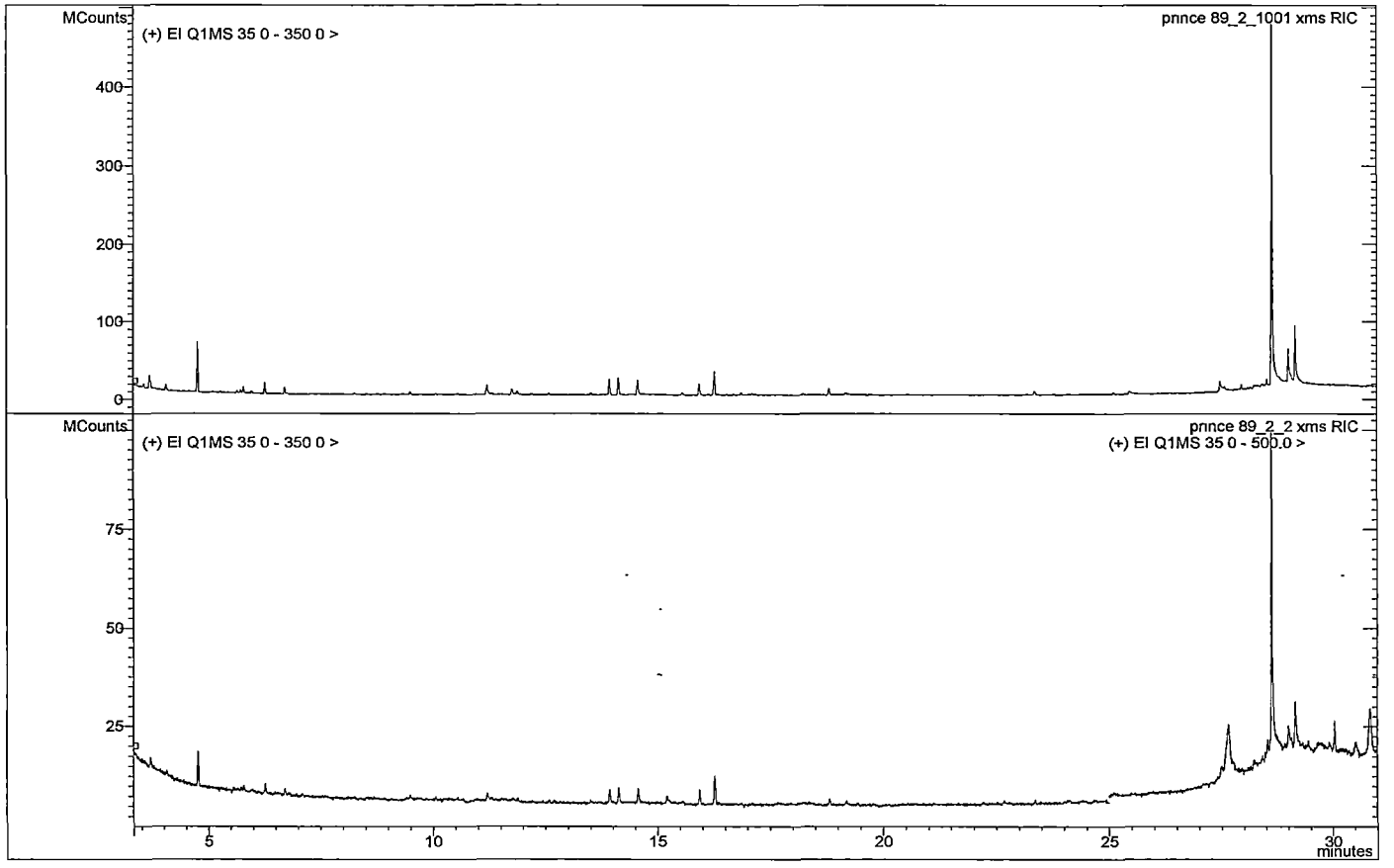
APPENDICES

GC-MS traces of the solvent extracts obtained from different parts of *C. rhomboidea* and *C. oblonga*

Chromatogram Plots



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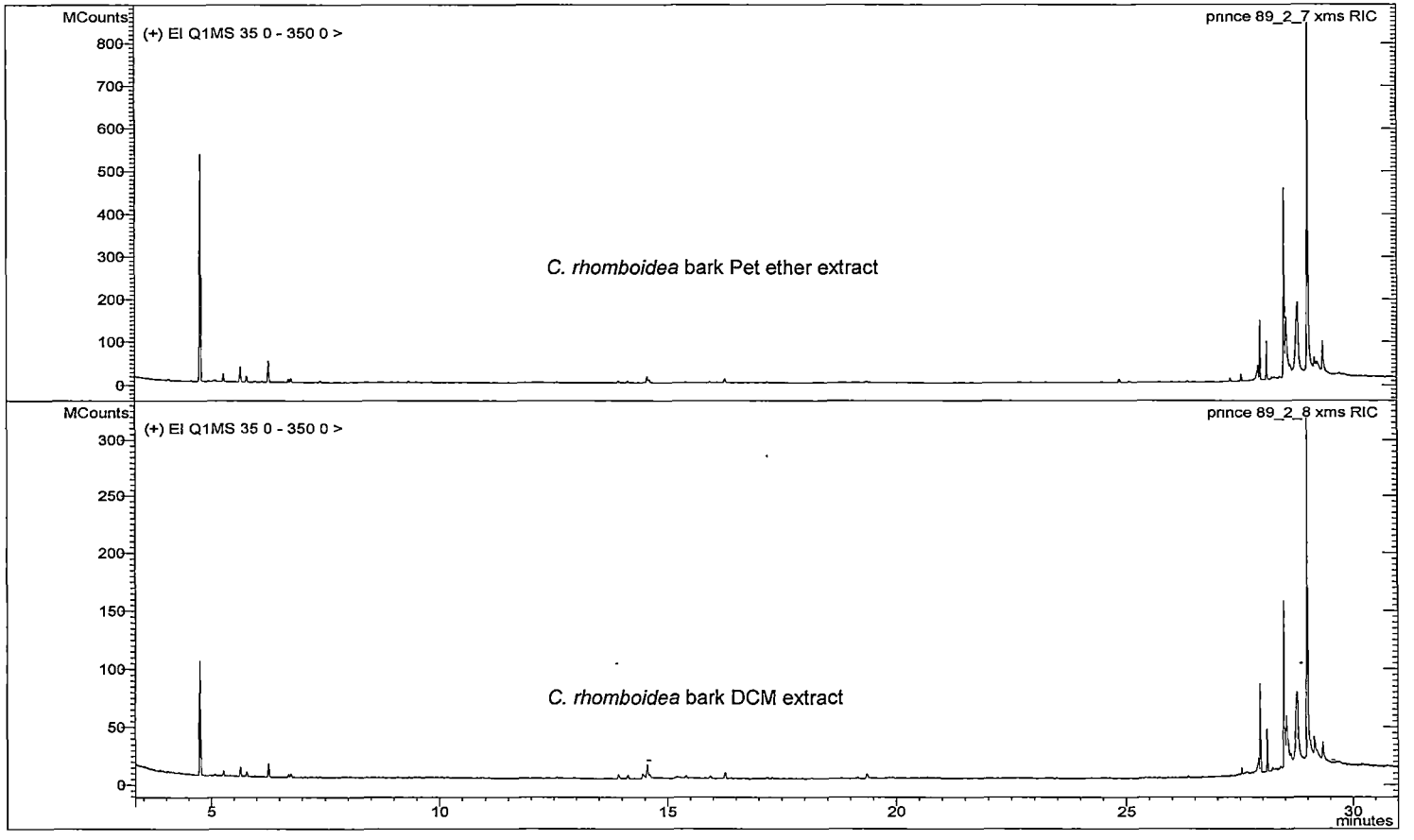

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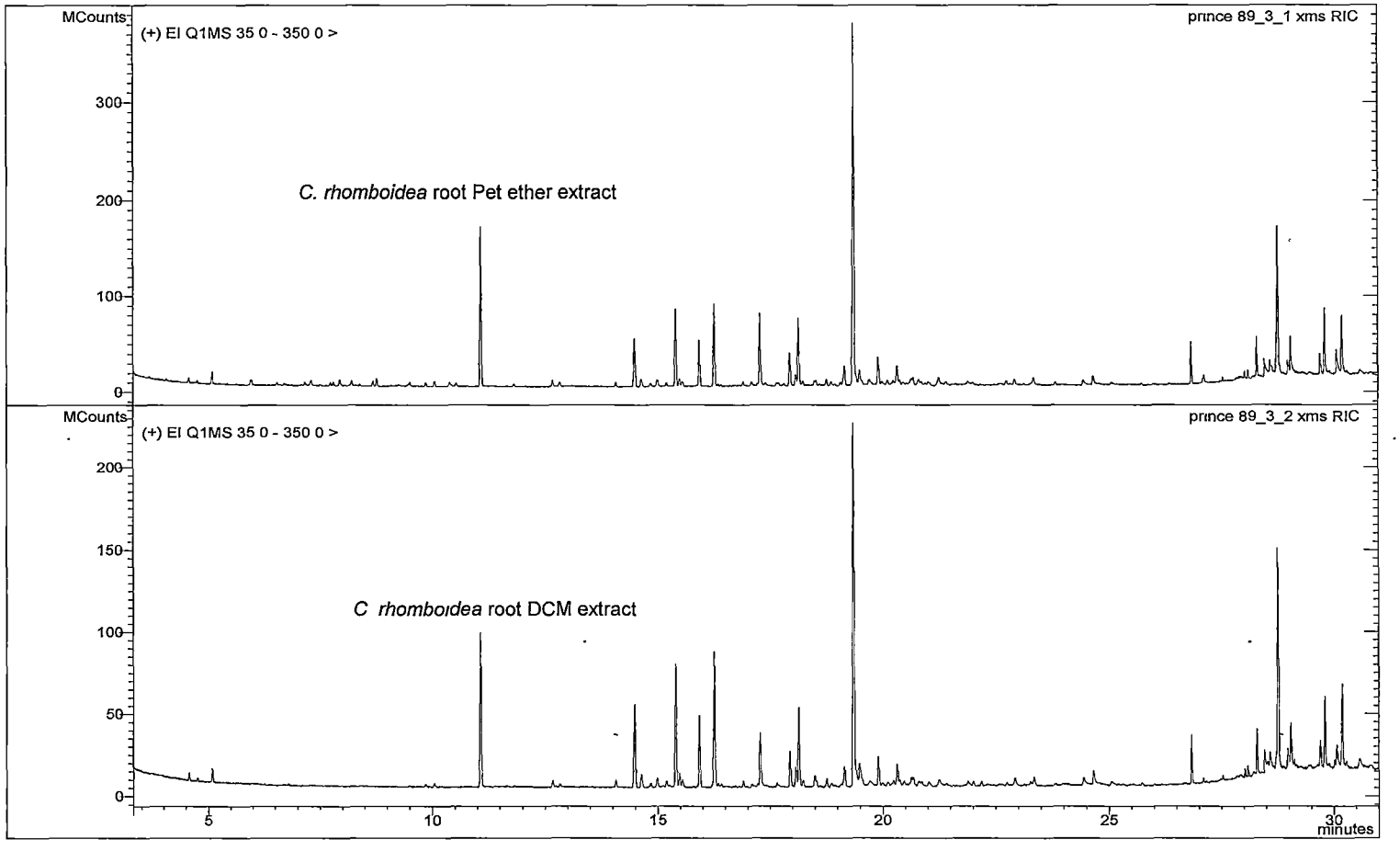

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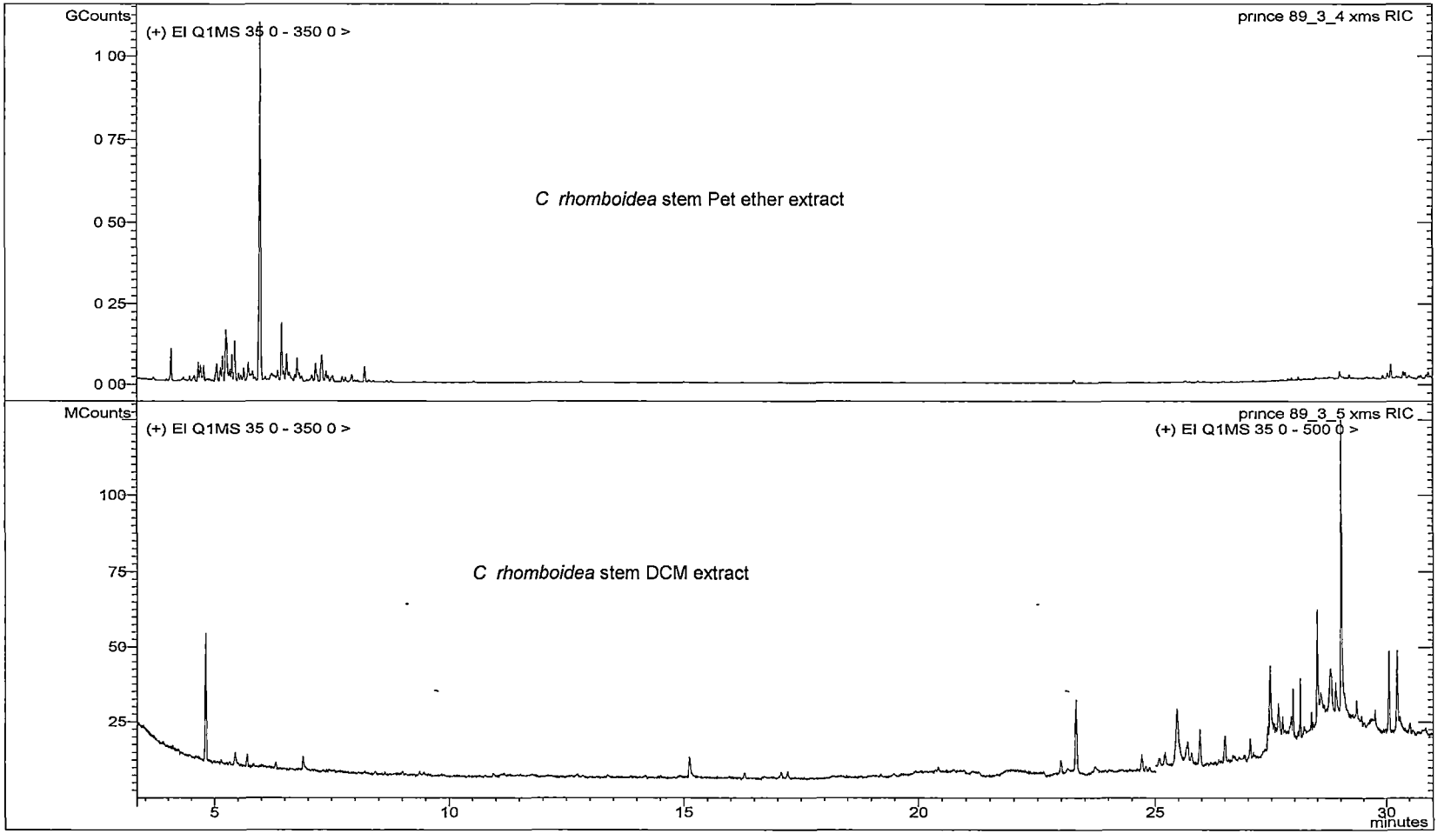

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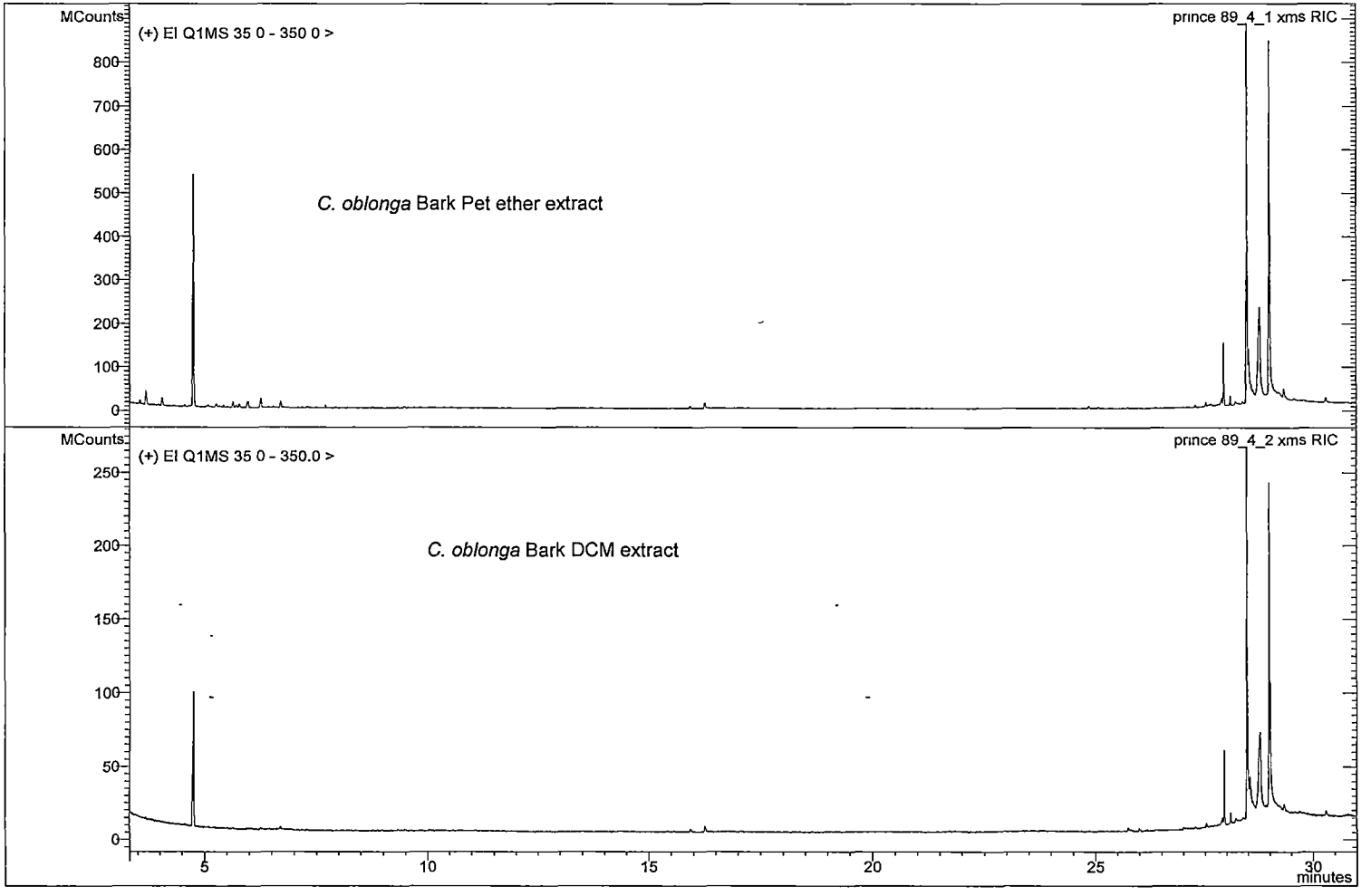

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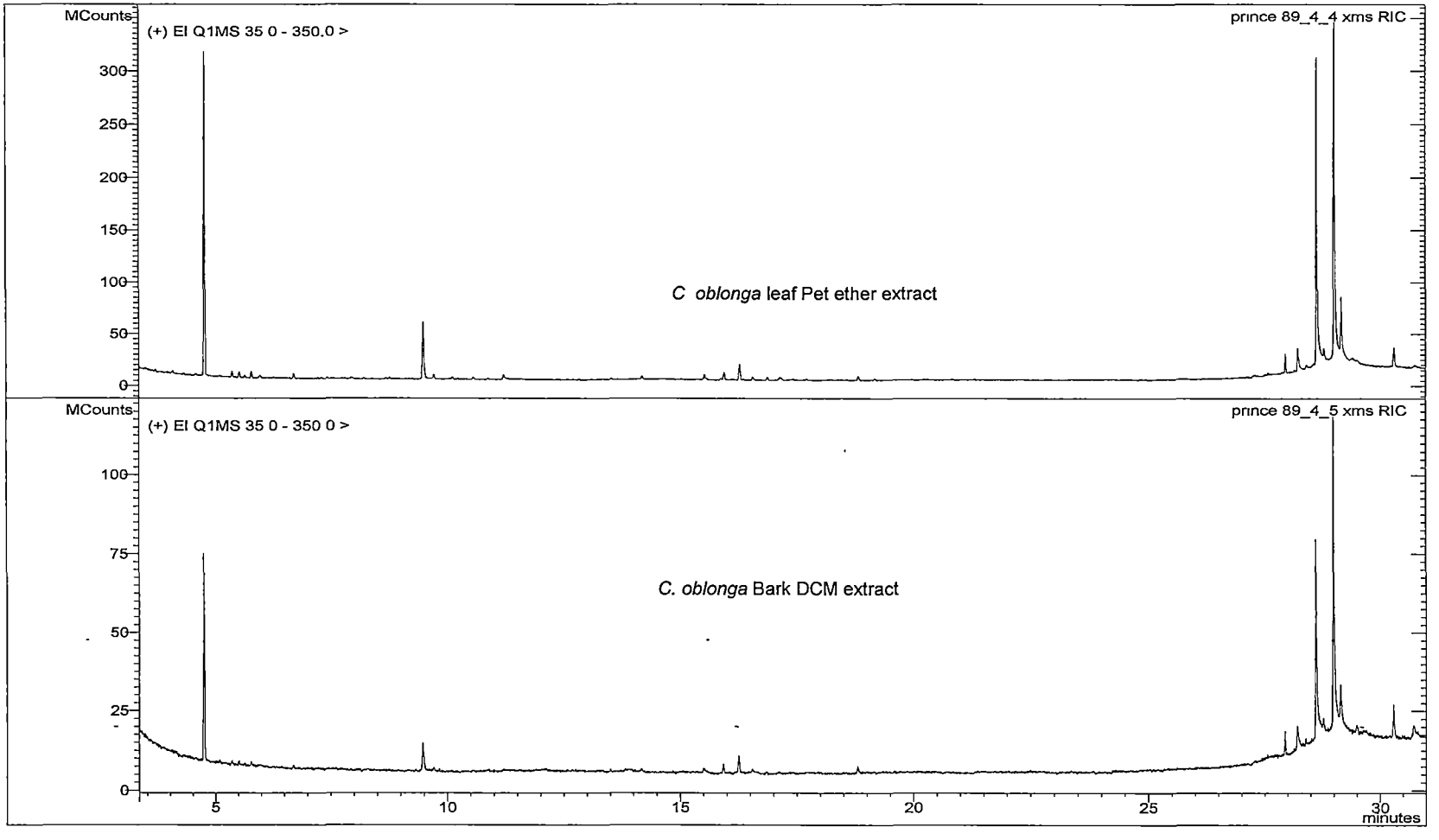

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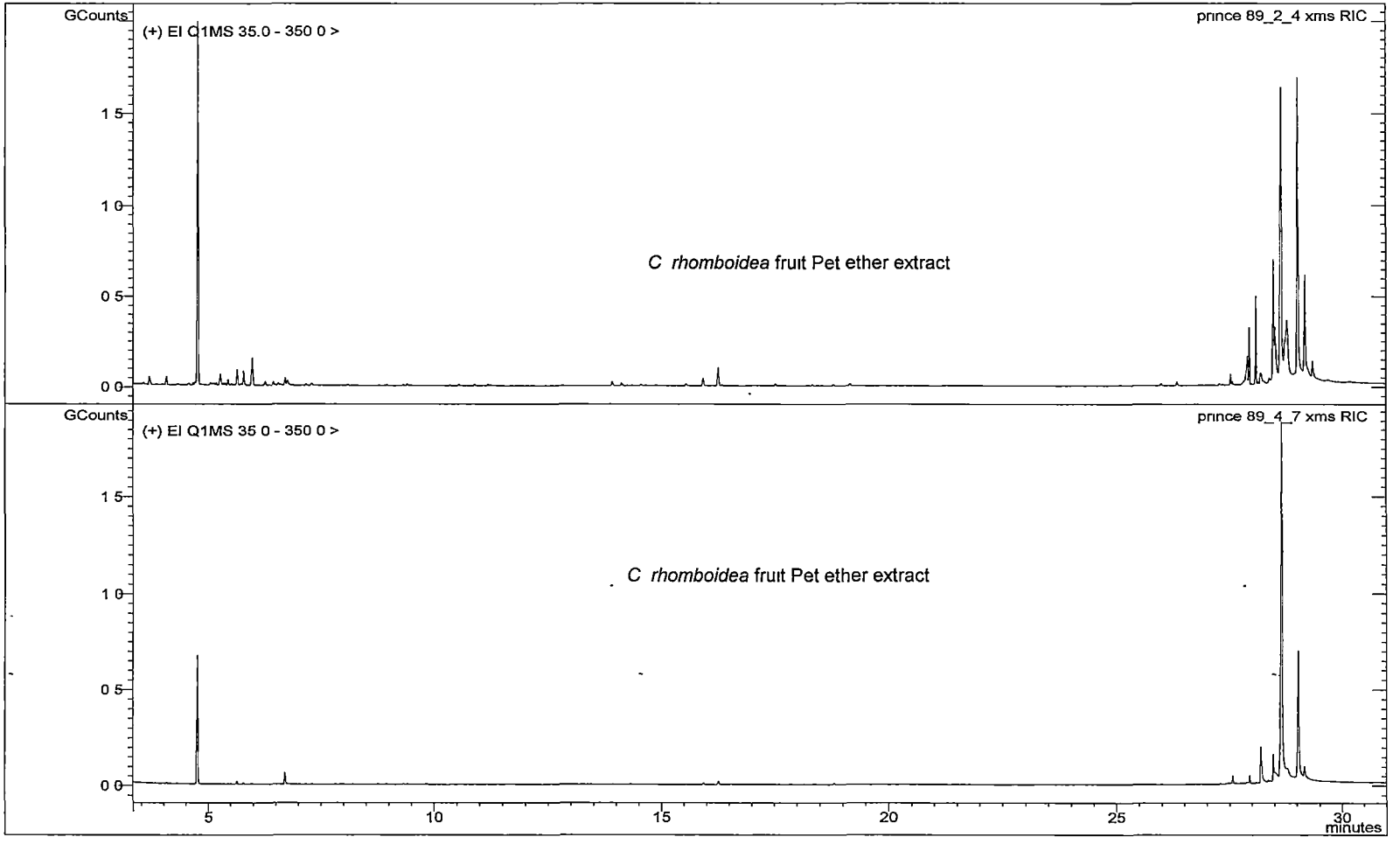

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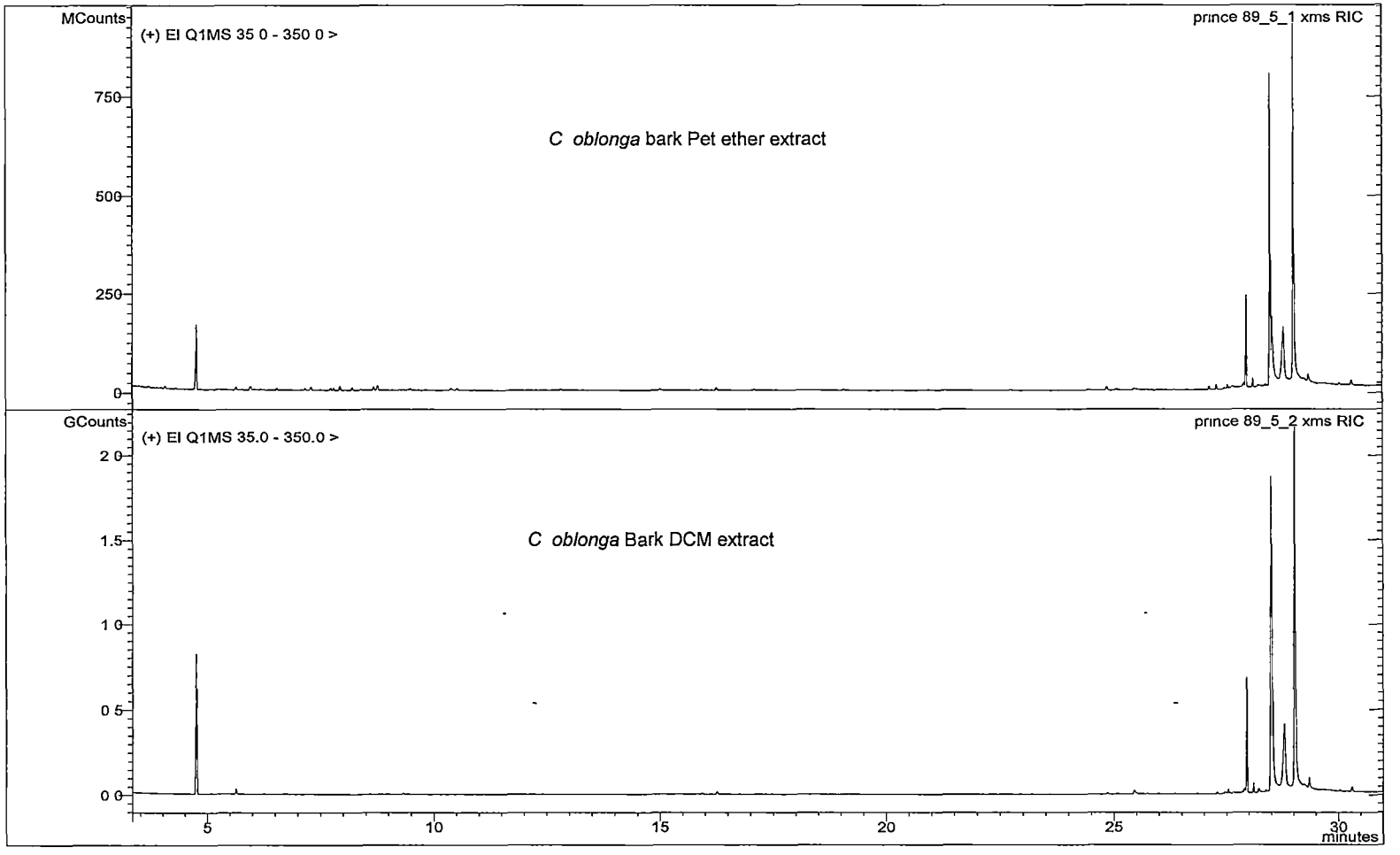

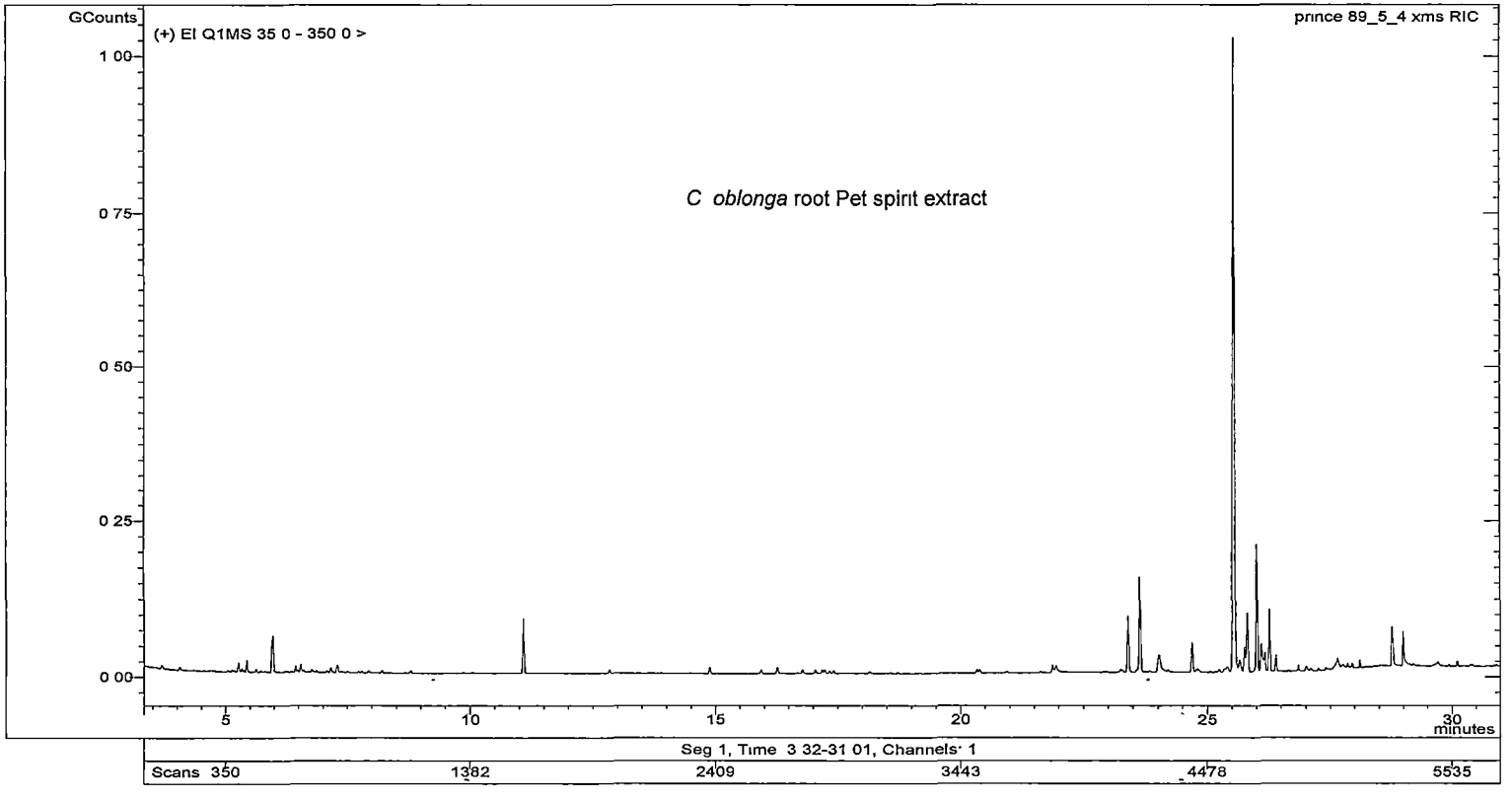
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minutes
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Scans 350
1382
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