Phytochemistry and Bioactive Properties of Plant Volatile Oils: Antibacterial, Antifungal and Antioxidant Activities

Hugh Jude Damien DORMAN BSc (Hons) MSc

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Aromatic & Medicinal Plant Biochemistry Group
Department of Biochemistry and Nutrition
Scottish Agricultural College
Auchincruive
Ayr, KA6 5HW
Scotland, UK

Declaration

"Through most of man's history, botany and medicine were, for all practical purposes, synonymous fields of knowledge, and the shaman, or witch-doctor - usually an accomplished botanist - represents probably the oldest professional man in the evolution of human culture."

R.E. Schultes, 1972,
Jeffery Professor of Biology and Director Emeritus,
Botanical Museum, Harvard University, Cambridge, Mass.

Nunc vos potentes omnes herbas deprecor,
exoro maiestatem vestrum, quas parens tellus
generavit et cunctis dono dedit.

A Roman prayer to all herbs.

Abstract

Aromatic and medicinal plants have been recognized since antiquity as possessing biological activities; chief amongst these are their antibacterial, antifungal and antioxidant properties. In this study, the chemical composition, the antimicrobial and *in vitro* antioxidant bioactivities the volatile oils extracted by hydrodistillation from aromatic and medicinal members of the plant families Geraniaceae: geranium (*Pelargonium graveolens* L'Herit); Lamiaceae: melissa (*Melissa officinalis* L.), monarda (*Monarda citriodora* var. *citriodora* Cerv. ex Lag.), oregano (*Origanum vulgare* ssp. *hirtum* (Link) Letsw.) and thyme (*Thymus vulgaris* L.); Myristicaceae: nutmeg (*Myristica fragrans* Houtt.); Myrtaceae: clove (*Syzygium caryophyllus* Gaertn.); Piperaceae: black pepper (*Piper nigrum* L.) and Umbelliferae: lovage (*Levisticum officinalis* L.) were investigated.

The chemical percentage composition of the volatile oils extracted from black pepper, clove, geranium, lovage (from both leaf and stem material), melissa, monarda, nutmeg, oregano and thyme were analysed using gas chromatography and mass spectroscopy. The findings of these analyses confirmed that volatile oils from aromatic and medicinal members of different plant families are principally mixtures of mono- and sesqui- terpenoids compounds. Furthermore, oil samples extracted from species of the same plant family, samples sourced from different parts of the same plant and a commercial and authenticated volatile oil described as from the same species may exhibit different compositions, i.e. the percentage composition and variation in individual phytochemicals.

A series of experiments were carried out in an attempt at assessing the antibacterial properties of black pepper, clove, geranium, melissa, nutmeg, oregano and thyme volatile oils and their main components. A collection of 25 test microorganisms [9 Gram-positive and 16 Gram-negative strains] was used throughout this study, representative of several major groups including human, plant and veterinary pathogens and food spoilage organisms. All the volatile oils demonstrated some degree of antiseptic activity with the oils of oregano and thyme being particularly active. The phenylpropanoid eugenol and the phenolic constituents carvacrol and thymol were found to be strongly antiseptic.

The antifungal activity of black pepper, clove, melissa, oregano and thyme volatile oils against the agriculturally important *Aspergillus* species *Aspergillus flavus* (Link) Fries and *Aspergillus niger* van Tieghen, and the *Fusarium* species *Fusarium culmorum* W.G. Smith was investigated. All the volatile oil samples demonstrated antifungal properties with variable degrees of efficacy across the concentration levels used in this study, with the volatile oils of oregano and thyme being particularly inhibitory.

The *in vitro* antioxidant properties of the volatile oils of black pepper, clove, nutmeg, oregano and thyme and their major components were evaluated by using a method routinely used in this laboratory, a simplified plate diffusion technique for determining lipid antioxidant activity using linoleic acid/β-carotene. To investigate further the potential antioxidant activity of these plant extracts and their main components and characterise their underpinning mechanism of action, an attempt to develop and optimize current antioxidant screening techniques was carried out. These methods included a thiobarbituric reactive species assay, a conjugated diene assay and a free-radical trapping assay.

Finally, a series of feeding trials were carried out to assess whether volatile oils feed at various concentrations prior to and during pregnancy can beneficially affect the compositional levels of major fatty acids in a maternal and foetal/neonatal model extracted from cholesteryl ester, triacylglyceride, free fatty acid and phosphoglyceride lipid fractions in an organ dependent manner. Particular focus centered upon the saturated fatty acids lauric, palmitic and stearic acids; the essential fatty acids linoleic [LA] (18:2*n-6*) and α-linolenic [ALA] (18:3*n-3*) acids and the long chain polyunsaturated fatty acid metabolic derivatives, e.g. arachidonic acid [ARA] (20:4*n-6*), docosahexænoic [DHA] (22:6*n-3*) and eicosapentænoic acid [EPA] (20:5*n-3*). The volatile oil of oregano was administered orally to female rats at 167mg Kg^{-1}, 334 mg Kg^{-1} and 843 mg Kg^{-1} concentration levels immediately prior to and during pregnancy to determine whether they affected the fatty acids composition in a variety of major maternal and neonatal organs. In addition to the aforementioned experiment, further feeding trails were carried out using the volatile oils of clove and nutmeg at a 50μg g^{-1} concentration level.

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Abbreviations

α-	alpha-
β-	beta-
Δ-, δ-	delta-
γ-	gamma-
sn-1, *sn*-2, *sn*-3	Denotes stereospecific numbering of carbon atoms 1, 2 and 3 respectively, of the trihydric alcohol glycerol
cis-	Groups on adjacent sides of a carbon-carbon double bond
trans-	Groups on the same sides of a carbon-carbon double bond
C_{18}	Fatty acids with 18 carbons
C_{20}	Fatty acids with 20 carbons
C_{22}	Fatty acids with 22 carbons
ALA	α-Linolenic Acid
ARA	Arachidonic Acid
DHA	Docosahexaenoic acid
DGLA	Dihomo-γ-linolenic Acid
EPA	Eicosapentaenoic Acid
GLA	γ-Linoleic Acid
LA	Linoleic Acid
LCPUFA	Long Chain Polyunsaturated Fatty Acid(s)
CE(s)	Cholesteryl Ester(s)
PL(s)	Phospholipid(s)
PUFA(s)	Polyunsaturated Fatty Acid(s)
TAG(s)	Triacylglceride(s)
ATP	Adenosine Triphosphate
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
BSM	British Standard Method
CHD	Coronary Heart Disease
CoA	Coenzyme A

EDTA	Ethylenediamine-tetra-acetic Acid
Cerv.	Cervantes
Gaertn.	Joseph Gaertner
Houtt.	Louis van Houtte
L.	Linnaeus
Lag.	Lagasca y Segura
Letsw.	Letswaart
Link	Johann Heinrich Friedrich
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectroscopy
HPLC	High Performance Liquid Chromatography
TLC	Thin Layer Chromatography
GSH	Glutathione
GSHPx	Glutathione Peroxidase
GSSG	Oxidised Glutathione
SOD	Superoxide Dismutase
AI%	% Antioxidant Index
H_2O_2	Hydrogen Peroxide
MDA	Malondialdehyde
RAE	Relative Antioxidant Efficiency
TBARS	Thiobarbituric Acid Reactive Species
SAC	Scottish Agricultural College
UV	Ultra Violet
YES	Yeast Extract Sucrose
%	Percent(age)
w/v	Weight for volume
v/v	Volume for volume
mL	Millilitre(s)
mg	Milligram(s)

μL	Microlitre(s)
μg	Microgram(s)
min(s)	Minute(s)

Dedication

This thesis is dedication to my parents.

Acknowledgements

I would like to acknowledge the major contributions made by the supervisors of this project, Dr Stanley G Deans and Professor Peter G Waterman. Furthermore, I would like to thank Professor Peter Surai and Professor Raymond C Noble for sharing the wealth of knowledge they have acquired in the areas of lipid peroxidation and lipid biochemistry.

I should also like to mention those individuals who have made significant contributions over the years, including M Baratt, Dr J Dixon, E Eaglesham, Dr H J Finlayson, K M^{ac}Issac, Dr E Simpson, Dr N Sparkes, F Thacker and Professor C Thomas.

Introduction

Plant volatile (essential) oils are important plant-derived products, principally composed of terpenoids [mono- (C_{10}), sesqui- (C_{15}) and some diterpenes (C_{20})]. Based upon the important biological isoprene or isopentenoid units, these oils occasionally contain components originating from other biosynthetic pathways, e.g. the shikimic acid pathway. They are stored in extracellular spaces in the epidermis or mesophylla and are not restricted to one specialized taxonomic group but are present throughout the plant kingdom.

Man has exploited these plant secondary metabolites since antiquity, with traces of a volatile oil industry dating from 5000 years ago (Verlet, 1993). Ancient Egyptians, who were the first exporters of perfumery products, using raw materials from Persia, India and China, assigned mystic and cosmic significance to these plant products while mass consumption of perfumes began with the Greeks who spread the use of volatile oils by conquest and barter. In the 17th Century, Grasse became the world capital of the perfumery industry, developing important technological advances including methods for extracting volatile components from plant materials and the cultivation of plants for oil extraction. Their earliest uses most probably would have been for culinary uses and perfumery, as volatile oil crops such as fresh or dried plant material rather than extracted oils. A medicinal role for plant material rich in volatile oil probably originates from around the same period. Research into plant volatile oils appears to have followed three investigative paths: the characterisation of their chemistry, the desire to elucidate their role in plant physiology and a search for new sources of compounds which may exhibit therapeutic activities.

Initially, the generation of volatile oils appeared to have no particular metabolic role and hence thought an expensive use of plant resources such as DNA, enzymes, photosynthate and energy. Investigations revealed that far from being an evolutionary error, they were actually responsible for a range of roles most notably as attractants, feeding deterrents, antibiotics, allelopathic agents, mediators of nutrient cycles and natural solvents (Deans and Waterman, 1993). The investigation into the beneficial activities of plant volatile oils has arisen from a discovery that higher plants may produce antibiotic compounds (phytoalexins) under certain circumstances which may demonstrate activity against human or veterinary pathogens, that matter of marine origin (e.g. algae) contain a variety and novelty of compounds, and the exploration

and evaluation of medicinal plant extracts with established human clinical experience in local medicines in African, Asian and South American countries. Ethnopharmacological studies have made substantial contributions to drug innovation by providing novel chemical structures and or mechanisms of action (Baerheim-Svendsen and Scheffer, 1982). A World Health Organisation estimation of the use of natural products as medicinal preparations in the developing world has been reported to be as high as 80% (Youdim, 1997).

Resurgence in interest into the bioactive properties of the volatile oils extracted from aromatic and medicinal plants has recently occurred. These have centered around fears regarding the efficacy of antibiotics currently used for the treatment of human and veterinary pathogens (e.g. the emergence of mupirocin-resistant methicillin-resistant *Staphylococcus aureus*), the increased demand for safer antimicrobial and antioxidant food preservatives which are more acceptable than the current preservatives whose safety in food stuffs is often questioned, the classification of GRAS status to essential oils and their components and the popularity of fringe medicines such as aromatherapy. In addition, early investigations suggest that plant volatile oils and their components (most notably phenolic terpenoids) may play a more direct biological role as antioxidants in the protection and metabolism of key polyunsaturated fatty acids (Simpson, 1995; Youdim, 1997), the prevention of degenerative disease including cardiovascular deterioration and in the processes involved in ageing (Youdim, 1997).

Numerous studies have shown the beneficial effects on important aspects of animal and human health of consuming increased amounts of *n-3* and *n-6* polyunsaturated fatty acids, in particular the long-chain *n-3* PUFAs eicosapentænoic and docosahexænoic acids (British Nutrition Foundation, 1992). Beneficial effects of these fatty acids have been observed not only in a range of animal and human disease states (Galli and Simopoulos, 1989), but also in the maintenance of optimal pre- and postnatal growth and development (Innis, 1991), validated by the high levels of long-chained PUFAs in vital organs of the developing neonate.

The aims of this study were to investigate the antibacterial, antifungal and antioxidant activities of the plant volatile oils and their components, where possible, from members of the Labiatae (Lamiaceae), Myristicaceae, Myrtaceae, Piperaceae and Umbelliferae families. In addition, to investigate the possible effects upon lipid

metabolism of the oral administration of selected volatile oils in maternal organs and in the case of oregano, neonatal organs.

1. Literature Review

1.1. Lipid Biochemistry

1.1.1. Definitions of Lipids

There are no exact, succinct nor agreed definitions of what lipids are (Christie, 1989; Gunstone and Herslof, 1992). A number of definitions for lipids have been put forward, including: ***a wide variety of natural products including fatty acids and their derivatives, steroids, terpenes, carotenoids and bile acids which have in common a ready solubility in organic solvents such as diethyl ether, hexane, benzene, chloroform or methanol*** (Christie, 1982); ***lipids are fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds*** (Christie, 1989); ***lipids are compounds based on fatty acids or closely related compounds such as the corresponding alcohols or the sphingosine bases*** (Gunstone and Herslof, 1992); ***actually or potentially compounds of the fatty acids*** (Mead and Alfin-Slater, 1986) and ***tissue components that are soluble in lipid solvents*** (Mead and Alfin-Slater, 1986). The term lipid may be used to describe ***compounds of biological origin that will partition into an organic solvent immiscible with water and differ from the other main components of tissues, the proteins and the carbohydrates*** (Hemming and Hawthorne, 1996a).

1.1.2. Fatty Acids

1.1.2.1. Structure and Nomenclature of Fatty Acids

Fatty acids have been defined as ***compounds synthesised in nature via the condensation of malonyl-coenzyme A units by a fatty acid synthetase complex*** (Christie, 1989). They are alkanoic or alkenoic acids and are the main components of most lipids (Gunstone and Herslof, 1992) and occur naturally in a combined form, principally as esters.

A fatty acid is composed of a carbon chain and a terminal carboxyl group. When each carbon in the chain, except the terminal ones, is bonded to two

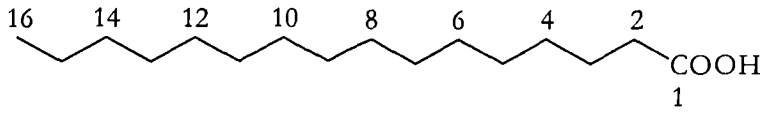
hydrogens, the acids are described as saturated. When each of two adjacent carbon atoms is bonded to only one hydrogen, there is an ethylenic double bond between the pair of carbons and the fatty acid is said to be unsaturated. If the chain contains only one double bond, it is a monounsaturated fatty acid and if the chain contains more than one double bond, it is a polyunsaturated fatty acid. Double bonds exist in two isomeric forms, *cis* and *trans*. Double bonds with a *cis* configuration possess hydrogen atoms on the same side of the double bond, thereby incurring a bend in the molecule. Those in the *trans* configuration contain hydrogen atoms on either side of the double bond, Figure 1.

Fatty acids are given systematic names which gives a complete description of their structure and degree of saturation. Saturated acids are given the chemical systematic name which denotes the number of carbon atoms, e.g. an eighteen-carbon straight chained saturated fatty acid is octadecanoic acid. The presence of a double bond is indicated by the change of the -anoic suffix to -enoic. Thus monounsaturated and polyunsaturated fatty acids can also be called monoenoic or polyenoic fatty acids (often shortened to monoenes and polyenes). An eighteen-carbon acid with one double bond is octadecenoic acid and with two double bonds is octadecadienoic acid. Unsaturated fatty acids are further defined by the position of the double bond closest to the methyl/terminal end of the molecule. Thus fatty acids with a double bond between carbon-6 and carbon-7 from the methyl end and those with a double bond between carbon-3 and carbon-4 from the methyl end are termed *n-6* and *n-3* fatty acids respectively. Similarly, fatty acids with *n-9* and *n-7* structural characteristics may be identified, Table 1. This distinction is of great importance as the position of the double bond in the carbon chain is critical to a fatty acid's biological activity.

Over the years a system of trivial names have grown up beside those of the more rigorous chemical nomenclature, i.e. the most widespread form of an octadecenoic acid is known as oleic acid and the most common form of octadecadienoic acid is known as linoleic acid. Furthermore, some fatty acids (DGLA, EPA, DPA and DHA) do not have trivial names and are generally referred to using the abbreviated form or other names. The British Nutrition Foundation (1992) has recommended the adoption of the shorthand chemical notation which recognizes the position of the terminal methyl double bond, e.g. α-linolenic acid would be

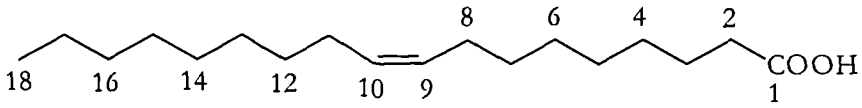
18:3*n-3*, indicating a fatty acid with 18 carbon atoms and three double bonds, Table 1.

Saturated

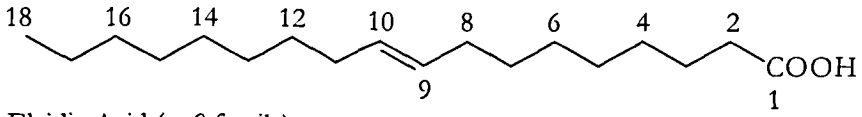


Palmitic Acid
Hexadecanoic acid

Monounsaturated

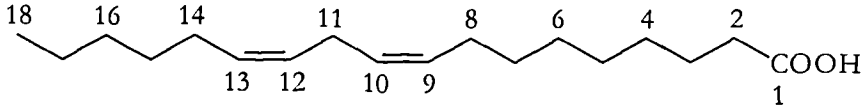


Oleic Acid (*n-9* family)
cis-9-octadecenoic acid (18:1 *n-9*)

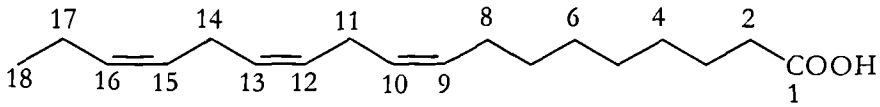


Elaidic Acid (*n-9* family)
trans-9-octadecenoic acid (18:1 *n-9*)

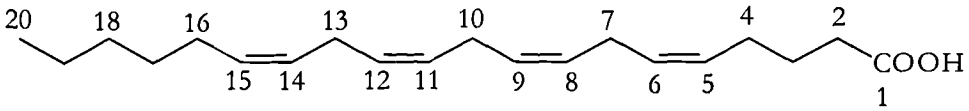
Polyunsaturated



Linoleic Acid (*n-6* family)
cis, *cis*-9, 12-octadecadienoic acid (18:2 *n-6*)



Alpha Linolenic Acid (*n-3* family)
all *cis*-9, 12, 15-octadecatrienoic acid (18:3 *n-3*)



Arachidonic Acid (*n-6* family)
all *cis*-5, 8, 11, 14-eicosatetraenoic acid (20:4 *n-6*)

Figure 1. Fatty acids: structure and nomenclature.

Table 1. A glossary of fatty acid nomenclature.

Systematic Name	Trivial Name/ Abbreviation	Shorthand Notation
Saturated		
dodecanoic	lauric	12:0
tetradecanoic	myristic	14:0
hexadecanoic	palmitic	16:0
octadecanoic	stearic	18:0
Monounsaturated		
Monoenoic		
cis-9-hexadecenoic	palmitoleic	16:1*n-7*
cis-9-octadecenoic	oleic	18:1*n-9*
trans-9-octadecenoic	elaidic	18:1*n-9*
cis-11-eicosænoic	gadoleic	20:1*n-9*
cis-13-docosænoic	erucic	22:1*n-9*
cis-11-docosænoic	cetoleic	22:1*n-11*
cis-15-tetracosænoic	nervonic	24:1*n-9*
Polyunsaturated		
Dienoic		
cis, *cis*-9, 12-octadecadienoic	linoleic	18:2*n-6*
Trienoic		
trans-5, *cis*-9, *cis*-12-octadecatrienoic	columbinic	18:3*n-6*
all *cis*-9, 12, 15-octadecatrienoic	α-linolenic	18:3*n-3*
all *cis*-6, 9, 12-octadecatrienoic	γ-linolenic	18:3*n-6*
9-*cis*, *trans*-11, *trans*-13-octadecatrienoic	α-eleostearic	18:3*n-9*
all *cis*-8, 11, 14-Eicosatrienoic	dihomo-γ-linolenic/ DGLA	20:3*n-6*
all *cis*-11, 14, 17-eicosatrienoic	mead acid	20:3*n-9*
Tetrænoic		
all *cis*-6, 9, 12, 15-octadecatetrænoic	stearidonic	18:4*n-3*
all *cis*-8, 11, 14-eicosatetrænoic	ETA	20:4*n-3*
all *cis*-5, 8, 11, 14-eicosatetrænoic	arachidonic	20:4*n-6*
all *cis*-7, 10, 13, 16-docosatetrænoic	adrenic	22:4*n-6*
Pentænoic		
all *cis*-5, 8, 11, 14, 17-eicosapentænoic	EPA	20:5*n-3*
all *cis*-7, 10, 13, 16, 19-docosapentænoic		22:5*n-3*
all *cis*-4, 7, 10, 13, 16, 19-docosapentænoic	DPA	22:5*n-6*
Hexænoic		
all *cis*-4, 7, 10, 13, 16, 19-docosahexænoic	docosahexænoic/DHA	22:6*n-3*

1.1.2.2. Synthesis of saturated fatty acids

All mammals are capable of synthesizing saturated fatty acids *de novo* from the simple precursors such as glucose or amino acids, using a fundamentally similar pathway (British Nutrition Foundation, 1992). The liver and the adipose tissue are the most significant organs for fatty acid synthesis, with species differences in the relative importance of these two organs. Long chain unsaturated fatty acids with more than three or four double bonds are produced by the elongation of the C_{18} unsaturated fatty acids by two carbon atoms and the insertion of another double bond between the carboxyl group and the first double bond.

Figure 2. Metabolic transformations of the three major unsaturated fatty acid families by desaturation and elongation. * The existence of 4-desaturase *in vivo* is doubtful (Sprecher, 1985).

Thus, after the elongation of the C_{18} unsaturated fatty acid by two carbon atoms, the enzyme 5-desaturase can insert a further double bond between carbon atoms 5 and 6 to yield arachidonic acid and eicosapentænoic acid respectively. After another elongation, the enzyme 4-desaturase can insert another double bond between carbons 4 and 5 to yield docosapentænoic acid (22:5*n-6*) and docosahexænoic acid (22:6*n-3*) respectively. This series of alternating elongations and desaturation produce a variety of LCPUFAs (Figure 2) necessary for membrane structure and eicosanoid production (Figure 3).

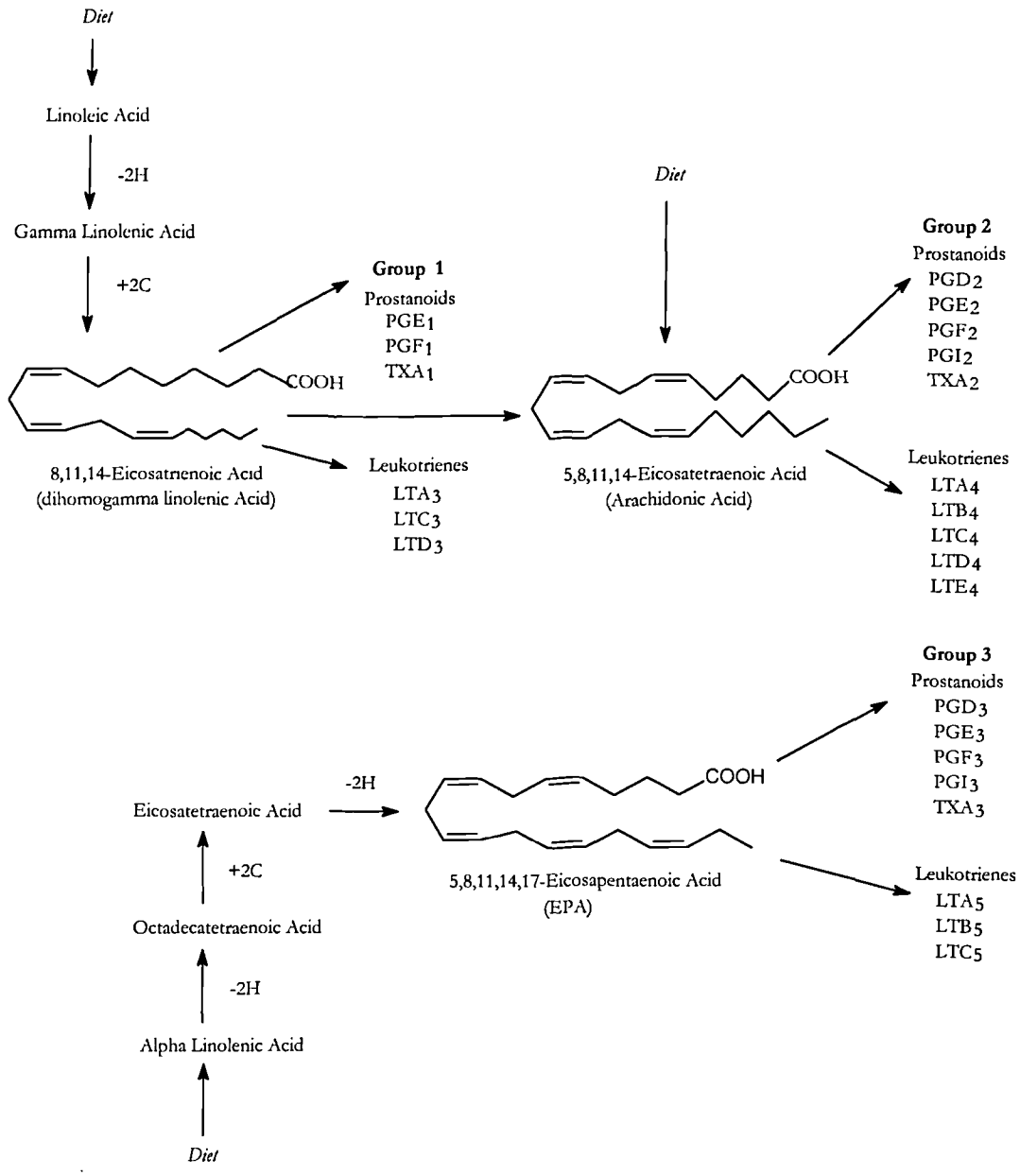


Figure 3. The three groups of eicosanoids and their biosynthetic origin (British Nutrition Foundation, 1992).

In summary, more than 1000 acids have been identified but the number occurring frequently in the common lipids is less than this. On the basis of the structures represented by these acids four generalizations can be made (Gunstone, 1992):

1. Most fatty acids are straight-chained compounds with an increasing even number of carbon atoms in each molecule. Chain lengths range from C_2 - C_{80} but the C_{12} - C_{22} members are the most common.

1. Monoene (monounsaturated) acids have a double bond with *cis* configuration, which usually occurs in one of a limited number of positions in the carbon chain.

2. Polyunsaturated acids have two or more *cis* double bonds each separated by one methylene group (-CH_2-). This means that the pentadiene unit occurs once or more times in most of these acids although other patterns of unsaturation are known.

3. Substituted acids are rare but no natural hydroxy and epoxy acids have been recognised.

1.1.3. Major Lipid Classes

1.1.3.1. Triacylglycerols

Triacylglycerols serve as a source of energy and reservoir of fatty acids for use in metabolic processes in animals and can be stored in practically unlimited amounts, unlike the limited storage capacity for carbohydrates and proteins. They are composed of a glycerol backbone esterified at the *sn*-1, *sn*-2 and *sn*-3 positions with a variety of fatty acids in a non-randomly distributed manner (Hemming and Hawthorne, 1996b). Furthermore, triacylglycerols are asymmetric in biochemical terms, thus positions *sn*-1 and *sn*-3 are distinguishable to the various enzyme systems (Brockerhoff, 1965). The chain length and the degree of unsaturation of the individual fatty acids making up the triglyceride determine its physical and chemical properties. Simple triacylglycerides of unsaturated fatty acids containing 10 or more carbons are solid at room temperature, whereas those with fewer than 10 carbons are usually liquid. Triacylglycerides containing only long-chain saturated fatty acids are

solid, whereas those containing a preponderance of unsaturated fatty acids are liquids.

Most of the information describing triacylglycerol composition of fats provides only the overall fatty acid composition. This type of data is important, but it does not reveal the position of the individual fatty acids on the glycerol moiety, a factor of significance in the absorption and utilization of fat. Therefore, the number of individual triacylglycerols becomes enormous.

1.1.3.2. Phospholipids

The term phospholipid is generally used to describe phosphoglycerides composed of a diacylglycerol conjugated to a substituent group composed of phosphoric acid and a base (Gunstone and Herslof, 1992). The principal bases are choline, serine and ethanolamine with the resulting phospholipids named phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine. As in the case of triacylglycerols, fatty acids of phospholipids have a distinguished positional distribution, with saturates acylated preferentially at the *sn*-1 position and unsaturated fatty acids at the *sn*-2 position. However, there is a good deal of exchange at these positions allowing phospholipids to be specifically manufactured for a particular function. The different arrangements of saturated and unsaturated residues in phospholipids arise from the fact that phospholipases preferentially attack the *sn*-2 position, unlike the neutral fat lipases. Unsaturated residues tend to be bulkier and longer than saturated ones and when incorporated into membranes, there may be a hole into which a hydrophobic portion of a polypeptide chain may fit.

The only common phospholipids which are not phosphoglycerides are sphingolipids (sphingomyelin) and cerebrosides based upon the amino alcohol sphingosine rather than glycerol which are found in nervous tissues (Gunstone and Herslof, 1992). The acyl residues are attached to these molecules via an amide rather than an ester link. The only phosphate containing derivatives of sphingosine are the sphingomyelins. These contain phosphoryl-choline and are found in plasma membranes and in the complex membranes of myelinated nerves. Unlike the other phospholipids, sphingomyelins contain mostly saturated fatty acids, some of them C_{20} to C_{24} in length. Sphingomyelin bilayars are thus more rigid than most others and

contain relatively little protein. Finally, cardiolipin is a phospholipid that is metabolically stable. It is found in the membranes of mitochondria.

1.1.4. Significance of n-3 *and* n-6 *Fatty Acids in Growth and Development*

Fatty acids are major constituents in triacylglycerides and phospholipids as well as other lipid fractions and have a number of important biological roles which are essential for normal development and human health throughout life. They act as an energy source, precursors of a range of metabolic regulators, help in the prevention and management of specific diseases and are important structurally and functionally in all animal tissues but especially during specific periods, e.g. foetal/neonatal development and ageing.

1.1.4.1. Essential Fatty Acids

Essential fatty acids (EFA) are defined as ***those fatty acids that are required for normal growth and physiological integrity and cannot be synthesized in adequate amounts by the body*** (British Nutrition Foundation, 1992) and ***fatty acids which are required by an organism for the maintenance of normal growth and reproduction, are not able to be synthesized by an organism and are therefore required in appropriate amounts in the diet*** (Leskanich and Noble, 1999). The *n-6* fatty acid regarded as essential in the mammalian diet is linoleic (18:2). Shortly following the identification of the essentiality of this fatty acid, an essential role for the *n-3* fatty acid α-linolenic (18:3) acid was identified, though with some degree of scientific debate (Chapkin, 1992).

Due to their importance with respect to human health, it has been suggested that the long-chain *n-3* and *n-6* polyunsaturated fatty acids arachidonic acid [ARA] (20:4*n-6*), docosahexænoic acid [DHA] (22:6*n-3*) and eicosapentænoic acid [EPA] (20:5*n-3*) should also be classified as "conditionally essential" (British Nutrition Foundation, 1992; Neuringer et al., 1988), being necessary to redress nutritional requirements occurring under certain specific conditions. Premature human infant birth is such an example, as the supply of placental long-chained PUFAs is curtailed at late gestation as the brain is undergoing rapid development and a supply and deposition of preformed long-chain PUFAs become critical to normal brain

development. At other times, the presence of sufficient amounts of the long-chain PUFA precursors linoleic and α-linolenic acid is considered sufficient for the formation of the longer chained metabolic derivatives.

1.1.4.2. Unsaturated Fatty Acids and Early Development

The long-chained PUFAs arachidonic, eicosapentænoic and docosahexænoic have more recently come to be regarded as essential to foetal and neonatal development due to their critical role in the normal growth and development of the brain and the retina (Innis, 1991a; British Nutrition Foundation, 1992). The brain requires significant quantities of both arachidonic and docosahexænoic acids at a period of rapid neonatal brain development know as the 'brain growth spurt'. A shortage in the supply of these fatty acids or their precursors, namely linoleic and α-linolenic acids, has been demonstrated to cause deleterious changes to the developing brain manifesting in reduced brain responsiveness and visual acuity (Neuringer *et al.*, 1988; British Nutrition Foundation, 1992; Carlson *et al.*, 1993a).

Furthermore, beneficial effect of dietary long-chain polyunsaturates on growth have been observed. These include weight gain in rats sucking dams fed on fish oil as opposed to maize oil (Yeh *et al.*, 1990) and plasma arachidonic acid associated with the phosphatidyl-choline fraction was found to be positively correlated with growth rate in the first year in premature human infants (Carlson *et al.*, 1993b). A positive and statistically significant correlation between human birth weight or head circumference and arachidonic acid or docosahexænoic acid in the phosphatidylethanolamine fraction of tissue sections taken from the umbilical cord (Crawford *et al.*, 1990) and the addition of arachidonic and docosahexænoic acids to preterm infant formula enhanced growth compared with control formula (Hansen *et al.*, 1997).

1.1.4.3. Unsaturated Fatty Acids and Ageing

It has been shown that ageing is associated with a marked decrease of long-chain PUFA levels with the resultant impact upon key cellular functions. With the loss of key PUFAs, especially arachidonate and docosahexænate, there is a concomitant loss of cellular membrane function and the onset of degenerative disease, such as age-related macular degeneration - a leading causes of visual

impairment. A review of the literature has recently been carried out which details age related changes in fatty acids, their effect upon human health and the significance diet and the potential of dietary supplementation in the management of these deleterious changes (Youdim, 1997).

1.1.5. Significance of n-3 *and* n-6 *Fatty Acids in Disease*

1.1.5.1. Unsaturated Fatty Acids and Coronary Heart Disease

Research into coronary heart disease has contributed to the identification of the risk factors associated with this major cause of death in the United Kingdom, however, the understanding of the aetiology of this disease is incomplete. Data available on the influence of unsaturated fatty acids and CHD is available from a variety of sources, Table 2. There appears to be no direct evidence of an association between monounsaturated fatty acid consumption and CHD risk. While the partial replacement of saturated fatty acids with *n-6* PUFAs may reduce the risk of a CHD event, there is no evidence of a reduction in the risk of death from all causes. In the case of *n-3* LCPUFAs, there appears to be evidence that the consumption of fish may be effective in the secondary prevention of CHD death.

1.1.5.2. *Unsaturated Fatty Acids and Atherosclerosis*

Atherosclerosis is characterized by an extensive thickening of the arterial intima that is associated with the migration of medial smooth muscle cells into the intima where they subsequently proliferate and produce excessive amounts of extracellular matrix. Many studies have indicated that hypercholesterolemia, with elevated levels of cholesterol-rich, low-density lipoprotein (LDL), is a major factor in the development of atherosclerosis (Özer *et al.*, 1996). Because of the nature of the risk factors involved in the aetiology of atherosclerosis, prevention through diet and particularly with reference to PUFAs has been investigated, The effect of *n-3* series of PUFAs and their influence upon the incidence of atherosclerosis is shown in Table 3.

1.1.6. *Significance of* n-3 *and* n-6 *Fatty Acids and Human Health other than Coronary Heart Disease*

1.1.6.1. Unsaturated Fatty Acids and Immune-System Mediated Inflammation

The dietary management of chronic diseases with a suspected immunological basis has been investigated, including dietary supplementation unsaturated fatty acids with the *n-3* PUFAs docosahexænoic acid and eicosapentænoic acids and the *n-6* PUFA found in sunflower seed oil and evening primrose seed oil, Table 4. Both types of unsaturated fatty acid treatments have reduced the pro-inflammatory effect, however, it still remains to be determined which form is preferable where there has been clinical benefit (British Nutrition Foundation, 1992).

1.1.6.2. Unsaturated Fatty Acids and Cancer

Cancer is only second to heart disease as a leading cause of death in the United Kingdom. The process of cancerous growth is considered to be initiated by either genetic, chemical or physical insult (Personal communication, Prof. P. Grasso), however, the precise cause of most cancers is unknown. Consequently, attention has focused upon dietary components as a source of initiators and promoters of carcinogenesis (British Nutrition Foundation, 1988), including the role of polyunsaturated fatty acids from both epidemiological and experimental studies (British Nutrition Foundation, 1992), Table 4.

The relationship between dietary lipids and colon, breast, prostate and pancreatic cancers have been briefly reviewed (British Nutrition Foundation, 1992). The strongest evidence between fats and cancer relates to colon cancer. Cross-country comparisons indicate that diets high in fat are associated with a greater risk of colon cancer than those containing less fat (Committee on Diet, 1982), later confirmed by Willet *et al.*, 1990. However, the epidemiological studies carried out relating to PUFA intake and human cancer appear inconclusive while evidence from animal models suggests that PUFAs of the *n-6* series are a necessary pre-requisite for the promotion and maintenance of tumour growth. Discrepancy between the human and animal studies may arise if *n-6* PUFAs show promotional effects in animals up to a certain threshold level, e.g. 4% of total energy. Consequently, a reduction in total

fat intake may decease the risk of developing cancer in addition to a change in the consumption of *n-6* fatty acids for increased levels of *n-3* fatty acids.

Table 2. Effects of *n-3* PUFA consumption upon coronary heart disease.

Effect Upon Blood Lipoprotein Profile
Plasma TAG and VLDL concentrations reduced (Sanders *et al.*, 1989; Molgaard *et al.*, 1990; Sanders and Hinds, 1992).
High fish oil intake (24g long chain *n-3* PUFAs day^{-1}) reduces concentration of LDL-cholesterol and apoprotein B (Harris, 1989).
Moderate fish oil intake increases HDL_2-cholesterol (Harris, 1989 and Sanders *et al.*, 1989).
Increase chylomicron clearance (Harris, 1989).
Increase in the proportion of small VLDL in humans (Sullivan *et al.*, 1986).
Reduction of Lipoprotein (a) in patients with elevated Lp(a) (Herrmann *et al.*, 1989).
Inhibition of TAG synthesis and apoB synthesis and secretion (Sanders *et al.*, 1985).
Effect upon Haemostatic and Circulatory Parameters
Reduced platelet adhesiveness and blood fibrinogen (Li and Steiner, 1990; Saynor and Gillot, 1988).
Increased PGI_3; decreased TXA_2 and TXA_3 in subjects with high basal levels (von Schacky *et al.*, 1985).
TXA_2 + TXA_3: PGI_2 + PGI_3 ratio shifted in Inuit (Fischer and Weber, 1986).
PGH_3 and TXA_3 produced from EPA are poor inducers of platelet activation (Needleman *et al.*, 1979).
Moderate intakes (~5g *n-3* PUFAs day^{-1}) reduce systolic and diastolic blood pressure 3-5mmHg (British Nutrition Foundation, 1992).
Reduced risk of ventricular fibrillation and sudden cardiac death (Gudbjarnason, 1989).
Reduction in angina (Saynor *et al.*, 1984).

Table 3. Effect of *n-3* PUFAs on the incidence of atherosclerosis.

Inverse dose-dependent relationship between CHD and fish oil consumption (Burr *et al.*, 1989; Dolecek *et al.*, 1989; Norell *et al.*, 1986; Dolecek and Grandits, 1991).
Reduction in restenosis in patients given fish oil (Dehmer *et al.*, 1988).
Regression of atherosclerosis in pigs, dogs and primates in hypercholesterolaemic state with dietary *n-3* long-chain fatty acids (Sassen *et al.*, 1989; Landymore *et al.*, 1985).

Table 4. Effect of *n-3* PUFAs upon human health other than coronary heart disease.

Effect Upon Immune System-Mediated Inflammation
Suppression of synthesis of tumour necrosis factor (TNF) and interleukin-1 - important mediator of immune reactions (Endres *et al.*, 1989).
EPA reduces LTB_4 production (pro-inflammation) (Prescott, 1984).
~1.1g GLA reduced prostaglandin E_2, LTB_4 and leukotriene C_4 produced by stimulated monocytes (Pullman-Mooar *et al.*, 1990).
EPA may improve SLE (lupus) (Westberg and Tarkowski, 1990).
Possible effect in slow progression of multiple sclerosis (British Nutrition Foundation, 1992).
Effect Upon Cancer
EPA inhibits catabolic activity of a tumour lipolytic factor by preventing cyclic AMP accumulation in fat cells (Tisdale and Beck, 1991).
Deceased expression of oncogenic *ras p*21 protein after culturing murine hyperplastic alveolar nodules with EPA (Telang *et al.*, 1988).
Inhibitory effect upon tumour induction in mammary gland and rat pancreas (Braden and Carroll, 1986; O'Connor *et al.*, 1985).
n-3 PUFAs inhibit the production of PGE_2 which accompanied colonic tumours (Minoura *et al.*, 1988).
Effect Upon Skin Disease
Possible reduction of acne (British Nutrition Foundation, 1992).
12g day^{-1} EPA resulted in a reduction of skin redness and scaling in psoriatic patients (Maurice, 1987).
18g day^{-1} EPA caused a reduction in itching and redness in psoriatic patients (Bittiner, 1988).
Evening primrose seed oil (7-10% GLA) produced clinical improvement in the skin of patients with atopic eczema (Bordoni, 1988)

In vitro studies, have suggested that unsaturated fatty acids, i.e. palmitoleic, oleic, linoleic and arachidonic acids, are more effective destroyers of tumour cells than their corresponding saturated acids (Siegal *et al.*, 1987), while PUFAs destroyed human breast, lung and prostate cancerous cells *in vitro* (Begin *et al.*, 1985). The most consistent and selective effects were obtained with fatty acids containing 3, 4 and 5 double bonds, e.g. GLA 18:3*n-6*, arachidonic acid 20:4*n-6* and EPA 20:5*n-3* (British Nutrition Foundation, 1992), Table 4.

1.1.6.3. Unsaturated Fatty Acids and Diabetes

In the United Kingdom, there are two major types of diabetes mellitus: insulin-dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM), which is more frequent amongst the obese. The risk of coronary heart disease is 2-3 times greater in diabetics than non-diabetics and microvascular complications such as diabetic nephropathy and retinopathy are also a problem. Although unsaturated fatty acids have not been implicated in the development of either of these conditions (Medalie *et al.*, 1975), the modulation by dietary means involving PUFAs has been the subject of a number of studies of people with this disease in terms of lipid metabolism, glucose and insulin metabolism and complication associated with this disease. The *n-3* series fatty acids have been demonstrated to be capable of reducing elevated levels of serum triacylglycerol, a possible risk factor for CHD in diabetes, usually as a mixture of EPA and DHA (Nestel *et al.*, 1984), while PUFAs of the *n-3* series do not appear to have a consistent effect upon cholesterol metabolism either in diabetic or non-diabetic subjects (Friday *et al.*, 1989 and Stacpoole *et al.*, 1989).

1.1.6.4. Unsaturated Fatty Acids and Skin Disease

The integrity and functionality of mammalian skin is dependent upon it's constituent unsaturated fatty acid composition. The influence of EFAs upon skin were demonstrated in a classical study carried out by Burr and Burr, 1929. EFA-deficiency results in excessive water loss, drying of the skin and the development of scaliness, haemorrhagic spots and cutaneous erythema. Subsequently, PUFAs of the epidermis have received much attention as these fatty acids are the precursors of the eicosanoids, which are important mediators of some major inflammatory skin diseases (Greaves, 1988). The potential of managing various skin diseases with an inflammatory basis, such as acne, psoriasis and atopic eczema, through dietary supplementation have been investigated with varying degrees of clinical success, Table 4.

1.2. Lipid Peroxidation

The direct reaction of a lipid molecule with a molecule of oxygen is termed autoxidation. Autoxidation has been defined as ***the slow, flameless oxidation of a***

material, generally by a radical-mediated chain reaction (Pryor, 1994) and is one of the oldest studied free radical chain reactions (Pryor, 1994). The term lipid peroxidation is often used interchangeably with autoxidation, as the principal products are generally lipid hydroperoxides. Unsaturated fatty acids incorporated into structural lipids and proteins are susceptible to attack by reactive oxygen species, which if uncontrolled may result in complex chemical changes precipitating dysfunctional modifications. There is increasing evidence implicating lipid peroxidation in the toxicity of a number of xenobiotics and the pathogenesis of many diseases (Kehrer and Smith, 1994), Table 5.

Table 5. Pathologies associated with free radical component (Kehrer and Smith, 1994).

Lung	**Eye**
Bronchopulmonary dysplasia	Retinopathy
Asbestos	Photic retinopathy
Emphysema	Cataracts
Iodopathic Pulmonary Fibrosis	
Heart and Cardiovascular System	**Skin**
Re-perfusion	Thermal Injury
Atherosclerosis	Contact Dermatitis
Haemochromatosis	Porphyria
Kidney	**Muscle**
Chemical insult: Heavy Metals	Muscular Dystrophy
Aminoglycosides	Multiple Sclerosis
Autoimmune Nephrosis	Exercise
Liver	**General/Miscellaneous**
Re-perfusion	Ageing
Endotoxin	Radiation Injury
	Inflammation
	Ischemia/re-perfusion
Blood	
Protoporphyrin	
Malaria	
Various Anaemias	

1.2.1. Mechanism of Lipid Peroxidation

The discovery of this phenomenon and its significance in susceptible media has led researchers to investigate the mechanisms underlying the processes involved. The peroxidation processes can be defined by three stages: initiation, propagation and termination, Figure 4.

1.2.1.1. Initiation

The oxidation of an unsaturated fatty acid gives rise to two free radicals: an alkoxyl radical ($L^{\cdot}$) and an $H^{\cdot}$ radical. The alkoxyl moiety may arise through the mediation of trace quantities of metal ions such as iron or copper, irradiation, light or heat. Hydroperoxides (LOOH), generated by a variety of pathways (i.e. the reaction of PUFAs with singlet oxygen or the lipoxygenase catalysed reactions), are normally present in lipid systems. They break down into alkoxyl, peroxyl and hydroxyl radicals by homolytic cleavage, hydrogen abstraction or bimolecular decomposition.

The free radicals generated from an allylic methylene group of an unsaturated fatty acid in the initiation phase tend to catalyze the next step in the oxidation process - propagation - and do not diffuse before reacting in their immediate vicinity.

1.2.1.2. Propagation

Propagation reactions are characteristic free radical reactions where a radical becomes responsible for the subsequent chemical transformation of numerous molecules into other reactive species, e.g. alkoxyls, hydroperoxides, peroxides, epoxides, alkenals, ketones and aldehydes. In the case of lipid-rich systems, the chain reactions consume oxygen resulting in the formation of different radical species. The species generated at this stage, unlike those from the initiation stage, are capable of diffusing along the plane of membranes thereby spreading the biochemical lesion. Hence, a reaction originating from an initial localized radical-lipid interaction may result in a cascade reaction causing disturbances throughout the cell, it's membrane and in some cases the extracellular domain. The products of these reactions initiate further chain reactions.

1.2.1.3. Termination

When the available PUFAs with susceptible double bonds sufficiently decrease, the reactive species generated in the propagation phase couple amongst themselves and no longer take a role in the chain reaction. Alternatively, once the supply of molecular oxygen is depleted, the reactive species react with either chain-breaking antioxidant components or proteins, the chain reaction ceases. These events result in the termination of the free radical-mediated chain reaction, where no further

reaction between reactive species generated in the initiation or propagation phase and susceptible moieties occurs.

1.2.2. Health Implications of Lipid Peroxides and Hydroperoxides

Coronary heart diseases, cancer, impact upon enzyme systems, pathology and ageing all have been linked to the by-products of the lipid free radicals. These species include lipid peroxides, oxidized cholesterol products and malondialdehyde.

1.2.2.1. Lipid Peroxides

Lipid peroxides are known to have bioactive properties and are implicated in pathological conditions. These include coronary heart disease (Sasaguri *et al.,* 1985), carcinogenicity (Fujimoto *et al.,* 1984; Addis, 1986), liver dysfunction (Kanazawa and Ashida, 1991), hypertrophy and teratogenicity (Cutler and Schneider, 1973). Other effects include anorexia, diarrhoea, diuresis, listlessness, oligodipsea, proteinuria and consequent reduction in body weight. They have been reported to inhibit ribonuclease, pepsin, trypin and pancreatic lipases *in vitro*, to attack low density lipoproteins (Jürgens *et al.,* 1986), to inhibit prostacyclin production and cause cellular damage and vacuolisation of endothelial cells.

1.2.2.2. Oxidised Cholesterol Products

The roles of cholesterol oxides, oxysterols and epoxides have been extensively investigated in connection with atherosclerosis and coronary heart disease. Specific oxysterols and epoxides have been reported to be mutagenic (Sevanian and Peterson, 1986) and carcinogenic (Suzuki *et al.,* 1986). These studies have indicated that the oxidation products of cholesterol and not the pure cholesterol itself are responsible for observed effects *in vitro* (Peng *et al.,* 1978) and *in vivo* (Imai *et al.,* 1980).

Cholesterol oxide is readily absorbed into high density lipoproteins, low density lipoproteins and very low density lipoproteins. *In vivo*, these oxides have been reported to inhibit the biosynthesis of cholesterol and decrease its incorporation into membranes (Peng *et al.,* 1985a) and have been reported to affect enzymes and transmembrane transport (Holmes and Yoss, 1984; Theunissen *et al.,* 1986).

1.2.2.3. Malondialdehyde

Malondialdehyde, is a secondary product of lipid peroxidation. The only limitation in the generation of MDA is that the fatty acid possesses three conjugated double bonds. The generation of MDA from the oxidation of linolenic acid is shown in Figure 4. It has been reported to be cytotoxic, mutagenic, capable of acting as both an initiator and a complete carcinogen *in vivo* (Shamberger *et al.,* 1974; Mukai and Goldstein, 1976; Bird *et al.,* 1982b; Basu and Marnett, 1983; Basu *et al.,* 1984; Basu and Marnett, 1984). *In vitro*, MDA has been demonstrated to cause vacuolisation, karyorrhexis, micronucleation and a reduction in protein synthesis in fibroblasts (Bird and Draper, 1980) and chromosomal aberrations in cultured mammalian cells (Bird *et al.,* 1982a).

1.2.3. Significance of Free Radical-Mediated Damage and Premature Neonatal Development

Under conditions of oxidative stress, the rate of free radical-mediated tissue damage is markedly increased (Russel, 1994). Research has established that premature infants are subjected to substantially more oxidative stress than term infants or adults (Bray and Levy, 1998) and have very marginal or deficient stores of many antioxidant compounds including vitamine E, β-carotene, GSH, ceruloplasmin, Se and Zn (Frank, 1991; Gopinathan *et al.*, 1994). Consequently, premature infants with a reduced antioxidant capacity may be highly susceptible to free radical-mediated damage when challenged with considerable oxidative stress. There is growing evidence implicating the role of free radicals in many of the diseases of the young and especially those associated with premature birth (Kelly, 1993). These include disorders affecting the eye, lungs and brain in premature infants, namely retinopathy of prematurity (Lucey and Dangman, 1984), bronchopulmonary dysplasia (Saugstad, 1996) and hypoxemic-ischemic brain injury (Varsila *et al.*, 1994) respectively.

Infants born prior to 30 weeks gestation or weighing less than 1500g at birth are often subjected to a sudden increase in free radical burden. The transition from a hypoxic placental environment to a relatively hyperoxic environment following birth represents a substantial oxidative stress. Moreover, because of their developmentally

immature lungs, premature infants often require treatment with hyperoxia in order to ensure adequate oxygen exchange between the atmosphere and the pulmonary blood supply. In addition to oxygen therapy, premature infants occasionally require intense drug therapy due to their vulnerability to opportunistic infection. Furthermore, the rates of catch-up, growth of premature infants during the first months are extremely rapid: utilization of oxygen to produce energy for growth is a free radical-producing process. Taken together, these observations seem to indicate that premature infants have a relatively high free radical burden and are subjected to a great deal more oxidative stress than term infants or adults.

1.3. Biologically Important Reactive Oxygen Species (ROS)

Reactive oxygen species are important mediators of cellular damage and extracellular injury. Membranes, lipids, lipoproteins, critical enzyme systems, proteins and ion channels are all susceptible to their unchecked generation. ROS is a collective term used to include oxygen derived radicals and non-radical derivatives of molecular oxygen (Halliwell, 1994). Oxygen-derived species may be a more appropriate term to describe these compounds than reactive as this is a relative term. Neither O_2^- nor H_2O_2 are particularly reactive in aqueous solution (Halliwell, 1994). The term oxidant is also used, however, both O_2- and H_2O_2 can act as oxidants and reductants in aqueous solution (Halliwell, 1994). ROS include oxygen, superoxide, hydroxyl radicals, hydroperoxides, hydrogen peroxide and certain xenobiotics.

1.3.1. Oxygen

The element oxygen (O) exists in the atmosphere as a molecule (O_2) and is known as either molecular oxygen or dioxygen. Ground state triplet dioxygen has two electrons possessing equal energy which occupy similarly shaped orbitals with identical energies on each oxygen atom. It can therefore be described as a di-radical, possessing two unpaired electrons with parallel spin. Reactions involving triplet oxygen with spin paired substances will yield another di-radical or two radicals. Molecular oxygen is unable to accept two electrons directly because the addition of a pair of anti-parallel electrons is restricted in the spins-parallel ground state. There is

very little inherent stabilization energy to drive such a reaction, consequently ground state oxygen is very unreactive in a biological matrix.

Molecular oxygen is capable of existing in two singlet states, $^1\Delta O_2$ ($^1O_2$) and $^1\Sigma g^+$. The $^1\Delta O_2$ species is biologically the most important as the $^1\Sigma g^+$ species rapidly decays to the $^1\Delta O_2$ form, which has a limited lifetime of only 2-7μs, emitting light energy as it decays. $^1\Delta O_2$ can be generated from the ground state triplet form: 23kcal/mol can pair the spins of its two electrons and place them into the same orbital. It can be generated on the skin by the absorption of electromagnetic energy or by porphyrins that may accumulate in some forms of porphyria and plays an important role in biological systems (Straight and Spikes, 1985; Pryor, 1986).

Although $^1\Delta O_2$ is not classified as a radical, its anti-parallel valence electron spins have no spin-restrictions on reactivity and can act as a two electron oxidant for many biomolecules (Fridovich, 1976; Chance *et al.,* 1979; Koppenol and Liebman, 1984; Pryor, 1986; McCord and Fridovich, 1988).

The "Oxygen Paradox" relates to oxygen's importance in aerobic metabolism due to its redox chemistry and its potential to cause cellular damage (Davies, 1995). The major role of oxygen for all aerobic organisms is simply to act as a sink or dumping ground for electrons (Davies, 1995). Over 95% of all the oxygen inhaled is involved in this role by undergoing a concerted tetravalent reduction catalysed by cytochrome oxidase (cytochrome c: oxygen oxidoreductase) of the complex IV in the mitochondrial electron transport chain.

The concerted tetravalent reduction of oxygen by the mitochondrial electron transport chain resulting in the production of water is considered safe. However, univalent reduction of oxygen generates intermediates with reactive characteristics (Davies, 1995). These include hydroxyl radical, superoxide anion radical and hydrogen peroxide reactive moieties.

Whether singlet oxygen is a major tissue damaging species in the body appears to be uncertain. However, during the peroxidation of membrane lipids, singlet oxygen or perhaps a closely resembling species, is produced and could potentiate further lipid peroxidation of PUFAs (Halliwell, 1990).

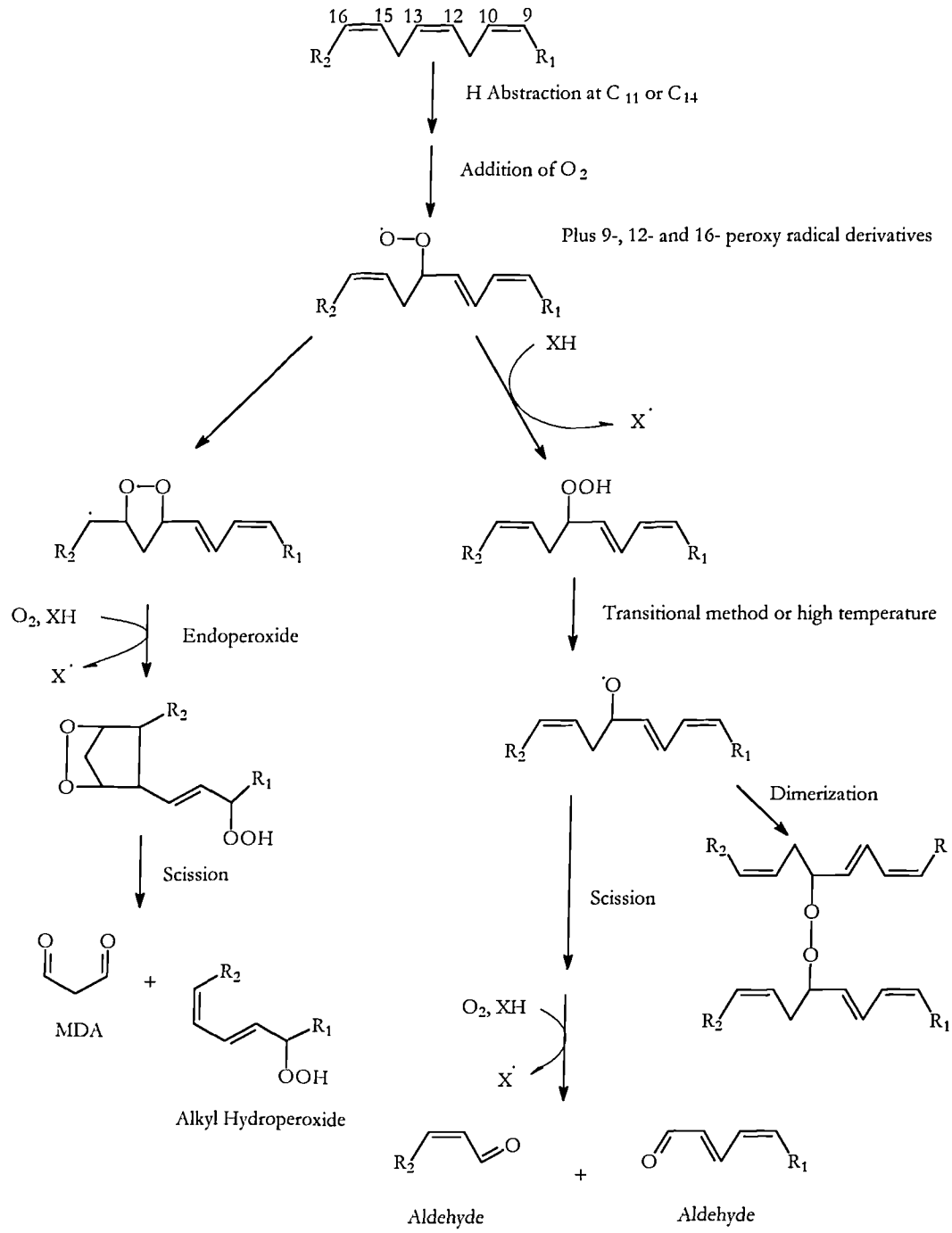


Figure 4. Initiation and secondary reactions for the oxidation of linolenic acid (Parkin and Damodaran, 1993).

1.3.2. Superoxide

The radical anion superoxide ($O_2^{\cdot -}$) is the one electron reduction product of oxygen (Halliwell, 1994; Davies, 1995), formed when one electron enters one of the

II *2p orbitals of oxygen (Gutteridge, 1994). It is known to be formed legitimately *in vivo* but also by 'chemical accident' (Fridovich, 1983; Fridovich, 1986; Halliwell and Gutteridge, 1989; Halliwell *et al.,* 1992). It is less reactive than the hydroxyl radical but the protonated form, the hydroperoxyl radical ($HO^{\cdot}_2$), is a powerful oxidant and reductant at least *in vitro* (Halliwell *et al.,* 1992), but little is stable at physiological pH (Halliwell *et al.,* 1992; Gutteridge, 1994; Halliwell and Cross, 1994).

The chemistry of the superoxide anion varies greatly depending upon its solution environment (Gutteridge, 1994). In aqueous solution, O^{-}_2 is a weak oxidising agent capable of oxidising molecules such as ascorbic acid and thiols but is a much stronger reducing agent reducing several iron complexes e.g. cytochrome C and ferric-EDTA. (Gutteridge, 1994). It rapidly disappears in aqueous solution due to its dismutation reaction in which hydrogen peroxide and oxygen are generated (Gutteridge, 1994).

It is probably the most important source of initiating radicals *in vivo* but under normal circumstances can not initiate lipid peroxidation (Pryor, 1994). Estimates of 1-2% of the total daily oxygen consumption go towards the mitochondrial generation of superoxide of which, for an average 60Kg woman maybe 160 to 320 mmol each day or for an average 80Kg man maybe 215 to 430 mmol based on an oxygen consumption of 6.4 L Kg^{-1} day^{-1} (Gutteridge, 1994).

At least two pathways exist in which superoxide can be converted to initiating radicals. In the first, iron-dependent Fenton chemistry, superoxide dismutates to form hydrogen peroxide which then is reduced by ferrous iron (or similar transition metals such as cuprous copper) to form the hydroxyl radical. In the second, superoxide can form potent oxidising agents by reacting with nitric oxide to give peroxynitrous acid (Pryor, 1994).

Superoxide may react very rapidly with a number targets including bacterial iron-sulphur proteins and associating enzymes essential to metabolism, e.g. aconitase (Halliwell and Cross, 1994). It has been claimed that superoxide in isolated sub-mitochondrial particles can inactivate the NADH dehydrogenase complex of the mitochondrial electron transport chain (Halliwell and Cross, 1994). Peroxynitrite, the product of $NO^{\cdot}$ and $O_2^{\cdot -}$ may cause direct damage by oxidising -SH groups (Halliwell and Cross, 1994). Additionally, at physiological pH it can protonate and decomposes

into a range of cytotoxic products including NO_2 - a powerful initiator of lipid peroxidation in biological fluids (Halliwell, 1994; Halliwell and Cross, 1994).

It is commonly held that the mitochondrial generation of the superoxide anion radicals represents the major intracellular source of oxygen radicals under physiological conditions (Chance *et al.,* 1979).

Another source of superoxide generation from oxygen and hydrogen peroxide is from autoxidation reactions involving compounds such as catecholamines, tetrahydrofolates and reduced flavins. The superoxide then oxidizes more of the compound, setting up a free radical chain reaction (Halliwell, 1994).

A large number of drugs undergo redox-cycling to generate superoxide radicals by being reduced by microsomal NADPH-cytochrome P_{450} reductase and other enzymes to semiquinoid radicals which react with molecular oxygen (Kappus, 1986). Ionizing radiation exposure *in vivo* will give rise to superoxide radicals by the reduction of dioxygen (Samokyszyn *et al.,* 1991).

1.3.3. Hydroxyl Radical ·OH

The most reactive radical is the hydroxyl radical (von Sonntag, 1987), reacting with almost all molecules found in living cells, with rate constants of 10^9-10^{10} M^{-1} s^{-1} (Anbar and Neta, 1967). It exhibits a half-life in nanoseconds (Samokyszyn *et al.,* 1991). The hydroxyl radical is a very powerful oxidising species and a strong electrophile, reacting unselectively. It may however show some specificity towards different sites of a molecule. It diffuses approximately 2 molecular diameters before reacting with surrounding molecules. It has been suggested that it reacts preferentially by addition rather than by outer sphere electron transfer. A general mechanism maybe as follows:

$$OH^{\cdot} + M \longrightarrow HO\dot{-}M$$

Figure 5. Reaction of a hydroxyl radical by outer sphere electron transfer.

The adduct HOM· may take two routes, either heterolysis with or without protonation of the OH group:

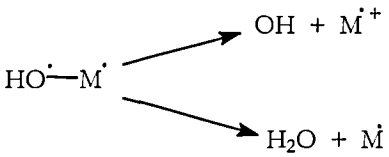


Figure 6. Routes for hydroxyl radical-molecule adduct dissociation.

The oxidized radical cation $M^{\cdot+}$ may then react with molecules and/or radicals. The damage caused by the superoxide radical and by hydrogen peroxide *in vivo* is thought to be due to their conversion into highly reactive oxidants, one of which is the hydroxyl radical.

1.3.4. Hydroperoxides/Hydrogen Peroxide

Hydrogen peroxide is the most common hydroperoxide and is generated by cellular biochemical processes (Ross and Moldeus, 1991). Monoamine oxidase, glycolate oxidase, urate oxidase amino oxidase and acid oxidase are among a number of flavin-linked oxidases capable of generating H_2O_2, In leukocytes, H_2O_2 is produced by the dismutation of superoxide by the enzyme superoxide dismutase (SOD; EC 1.15.1.1.). Although hydrogen peroxide is only mildly reactive at micromolar concentrations, at higher concentrations (>50 μM) it may attack certain cellular sites. It can oxidize an essential -SH group on the glycolytic enzyme *glyceraldehyde-3-phosphate dehydrogenase* and consequently block glycolysis and cellular energy metabolism. It readily mixes with water and is treated as such by the body. It has the ability to cross membranes and undergo metal catalysed reactions.

Hydroperoxides are produced in several metabolic pathways and may occur as an abnormal by-product of other reactions. Organic hydroperoxides are produced by the oxygenation of PUFAs catalysed by lipoxygenases and prostaglandin H synthetase. Hydroperoxides are split by components such as the cytochromes, haemoglobin and iron chelates into alkoxyl radicals which may react with PUFAs, thereby initiating and propagating lipid peroxidation. Cytochrome P_{450} may act as a peroxidase, resulting in the formation of a hydroxyl group (Kappus, 1991). Various iron complexes react with PUFA-derived and other hydroperoxides to yield alkoxyl and/or peroxyl radicals. These oxygen-centered radicals react with other PUFAs by H-atom abstraction, resulting in the propagation of lipid preoccupation. The

mechanism where non-haeme ferric or ferrous iron complexes catalyze homolysis of hydroperoxides to oxygen-centered radicals involves one electron oxidation to peroxyl radicals (Figure 7) or reduction to alkoxyl radicals (Figure 8).

$$LOOH + Fe^{3+} \longrightarrow LOO\cdot + Fe^{2+} + H$$

Figure 7. Ferric catalysed homolysis of hydroperoxides to form a peroxyl radical.

The alkoxyl radicals can react with PUFAs by H-atom abstraction (Figure 9), yielding new carbon centered radicals which may couple with dioxygen-yielding peroxyl radicals or more importantly, the PUFA hydroperoxide derived alkoxyl radicals may undergo intermolecular cyclisation (Figure 10). Finally, the alkoxyl radical may undergo β-scission reactions (Figure 11). The product of the β-scission reaction (a carbon centered radical) can couple with dioxygen-yielding peroxyl radicals or react with PUFA unsaturated double bonds to produce alkanes.

$$LOOH + Fe^{2+} \longrightarrow LO\cdot + Fe^{3+} + HO$$

Figure 8. Ferrous catalysed homolysis of hydroperoxides to form an alkoxyl radical.

$$LO\cdot + LH \longrightarrow LOH + L$$

Figure 9. Alkoxyl radical reaction with a PUFA by H-atom abstraction.

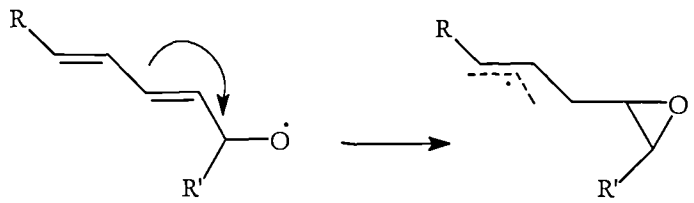


Figure 10. Intermolecular cyclisation of PUFA hydroperoxide derived alkoxyl radicals.

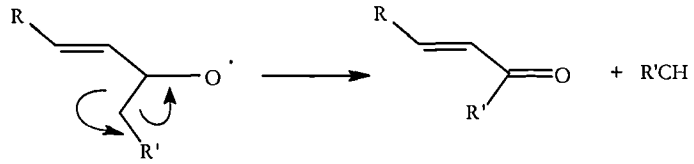


Figure 11. β-scission reaction of an alkoxyl radical.

It should be noted that lipid hydroperoxides are also enzymatically generated from arachidonic acid in a variety of cells. These peroxides include the cyclic endoperoxide intermediates formed by cyclo-oxygenase, PPG_2 and PGH_2 and the hydroperoxy eicosatetrænoic acids (HPETE's) formed by lipoxygenase (Kappus, 1991).

1.4. Antioxidants and Their Mechanism of Action

An antioxidant maybe defined as ***any substance that, when present at low concentrations compared to those of an oxidizable substrate such as proteins, lipids, carbohydrates and DNA, significantly delays or prevents the oxidation of that substrate*** (Halliwell, 1990; Gutteridge, 1994). Chemicals with antioxidant properties are often compounds capable of donating hydrogen radicals, thereby reducing the primary radicals to a non-radical chemical species while they become transformed into oxidized antioxidant radicals. Significantly, antioxidants converted into radical species have very low reactivity or are rapidly recycled into a non-radical.

Antioxidants important in the maintenance of the oxidative balance in living systems may be broadly categorized as *in vivo* endogenous or dietary antioxidants. Endogenous antioxidants include extracellular (Forni *et al.,* 1983; Dimascio, 1991a; Dimascio *et al.,* 1991b; Ross, 1988; Ross and Moldeus, 1991; Gutteridge and Quinlan, 1993; Krsekstaples and Webster, 1993; Corchia *et al.,* 1994; Grant *et al.,* 1994; Gutteridge, 1994; Vercellotti *et al.,* 1994; Decossin *et al.,* 1995; Powers, 1995; Reiter *et al.,* 1995) and intracellular compounds, enzymes and metabolic end products (Ames *et al.,* 1981; Pasantesmorales *et al.,* 1984; Pasantesmorales *et al.,* 1985; Stocker *et al.,* 1987a; Ross and Moldeus, 1991; Gopinathan *et al.,* 1994). Dietary antioxidants include both naturally occurring compounds from plant and marine sources and synthetic compounds.

Irrespective of the origin of these compounds, antioxidants can be categorized by their general activity (primary or chain-breaking, secondary or preventive and synergistic antioxidants) or by their chemistry or specific activity both *in vitro* and *in vivo*.

1.4.1. Primary or Chain Breaking Antioxidants

Primary antioxidants appear to act either by donating hydrogen radicals or electrons to radical species thereby converting them into a non-radical moiety and hence terminating the free radical chain reaction during initiation or propagation, Figure 12.

The second mechanism involves the antioxidant reacting with a lipid radical to form a lipid - antioxidant complex (Rajalakshmi and Narasimhan, 1995), Figure 13. This mechanism is significant at very low oxygen pressures or very low rates of chain initiation or in the presence of primary antioxidants at high concentrations (Gordon, 1990).

1.4.2. Secondary or Preventive Antioxidants

These classes of compounds retard the rate of chain initiation by removing or decreasing the concentration of local molecular oxygen, scavenging singlet oxygen or decomposing hydroperoxides, sequestering trace metal ions which promote initiation reactions or by absorbing UV energy. They usually require the presence of other components, e.g. synergists for effective activity.

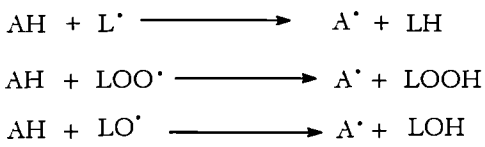


Figure 12. Hydrogen donation to radical species to form non-radical moieties.

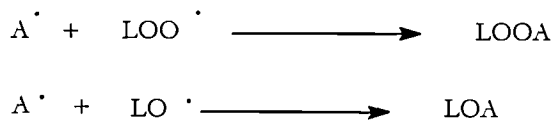


Figure 13. Formation of a lipid-primary antioxidant complex.

1.4.3. Synergistic Antioxidants

Synergists maybe defined as substances enhancing the activity of primary antioxidants, without being antioxidants themselves, usually acting by electron donation, chelation or oxygen scavenging (Kikugawa *et al.*, 1990). Although synergists may work via different mechanisms, they tend to extend the active life of a primary antioxidant and often increase their activity when combined and are not to be destroyed by the generated radicals.

1.4.4. Phenolics

Under normal oxygen pressures, the commonest lipid radical is the alkyl peroxyl radical which is an oxidising agent. The alkyl peroxyl radical is readily reduced to an anion and a hydroperoxide by an electron donor or converted to a hydroperoxide directly by a hydrogen donor. In thus cases, a compound which rapidly donates a hydrogen atom to a lipid radical becomes a radical which is more stable than the lipid radical or is converted to other stable products may be described as a primary antioxidant. Numerous phenolic compounds act in this fashion and are the best known antioxidants.

The most important factor in phenolic antioxidants is the stability or reactivity of the primary antioxidant radical generated after hydrogen donation. The phenoxyl radical formed by the reaction of the phenol with a lipid radical is stabilized by the delocalization of an unpaired electron around the aromatic ring as indicated by the valence bond isomers (Ingold, 1960), Figure 14. In the case of the phenolic α-tocopheroxyl radical, the delocalization of the solitary electron over the aromatic ring is shown by way of various mesomeric structures (Jadhav *et al.*, 1995).

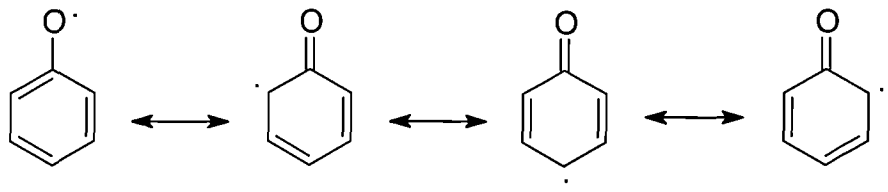


Figure 14. Valance bond isomers of a phenol radical formed by reaction of a phenol with a lipid radical. (Gordon, 1990).

A phenoxyl radical is further stabilized by bulky substituents in the 2 and 6 positions as in the case of BHT. By increasing the steric hindrance one reduces possible propagation reactions involving the antioxidant radical (Gordon, 1990), Figure 15.

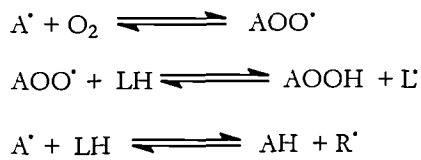


Figure 15. Possible propagation reactions involving a primary antioxidant radical.

The capacity of hydrogen donation works by inductive effect which is a function of the nature and the number of substituent groups attached either directly to the aromatic ring, e.g. BHT, or attached away from the structure of the compound, e.g. carnosic acid. Substitution of alkyl groups at the 2,4 and 6-positions of the aromatic ring increases the electron density by their inductive effect on the hydroxyl moiety and which causes an increase in its reactivity with lipid radicals. Furthermore, substitution at the 4-position with either an ethyl or *n*-butyl group rather than a methyl group improves the activity greatly. However, the substitution of either a longer alkyl group or a branched group in this position reduces the activity (Ingold, 1960).

The presence of bulky groups in the vicinity of the hydroxyl group also affects the ability of the hydrogen donating hydroxyl group of these compounds. The substitution of bulky groups at the 2 and 4-positions reduces the rate at which the phenol reacts with lipid radicals due to the steric hindrance effect these groups confer (Gordon, 1990).

The effective phenolic antioxidant concentration in an oxidative system appears to be dependent upon the structure of the compound, the conditions of the oxidation and the lipid system. *In vitro*, phenolic antioxidants are effective at increasing the induction period when the lipid system has not undergone sever deterioration, while appearing to be virtually ineffective in severely deteriorated systems (Mabrouk and Dugan, 1961; Scott, 1965). In addition, phenolic antioxidants at high concentration may demonstrate pro-oxidant effects as they may take part in the peroxidation initiation reactions (Lundberg *et al.*, 1947).

1.4.5. Radical Trapping Agents

Carotenoids and particularly β-carotene have been studied for their antioxidant properties at low oxygen pressures (Burton and Ingold, 1984). There appears to be two mechanisms for the activity of β-carotene. Firstly, this carotenoid prevents the formation of hydroperoxides in the presence of singlet oxygen by quenching (trapping) this radical (Foote and Denny, 1968; Foote, 1976). It appears that β-carotene is not destroyed during the quenching process and can repeat the process with additional singlet oxygen molecules (Deshpande *et al.,* 1995). The ability of the carotenoids to quench singlet oxygen appears to be dependent on the number of double bonds in the molecule. Carotenoids with 9,10 or 11 conjugated double bonds are reported to be better than those with 8 or fewer conjugated double bonds (Deshpande *et al.,* 1995; Rajalakshmi and Narasimhan, 1995). Secondly, at low oxygen pressures and in the absence of singlet oxygen, β-carotene can react rapidly with chain-carrying peroxyl radicals, producing a resonance-stabilized carbon-centered radical (Burton and Ingold, 1984), therefore, acting as a primary antioxidant, Figure 16.

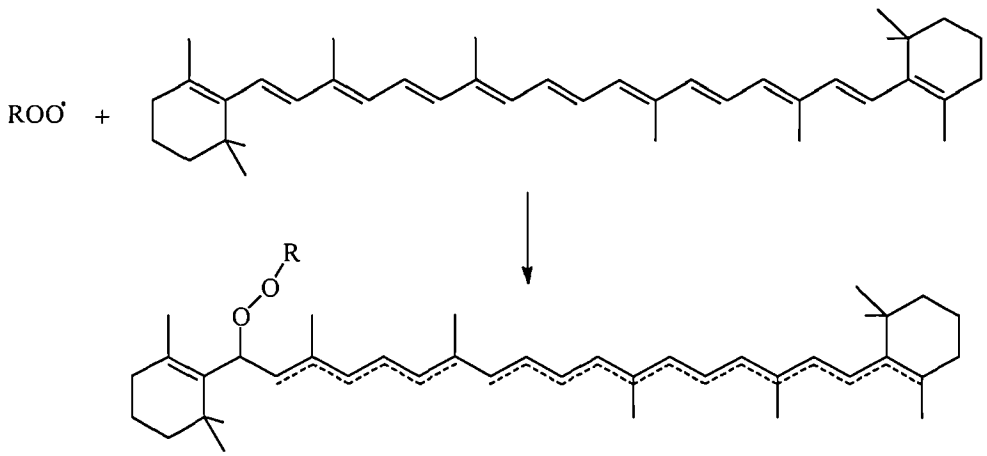


Figure 16. Reaction of β-carotene with an alkyl peroxyl radical (ROO.), forming a resonance-stabilized carbon-centered radical. (Burton and Ingold, 1984).

1.4.6. Chelating Agents

Chelating agents which form σ-bonds with metal ions are effective secondary antioxidants by reducing the redox potential and thereby stabilizing the oxidized

form of the metal and consequently preventing its involvement in free radical chain reactions. Chelating agents which form π-bonds, e.g. heterocyclic bases, have a pro-oxidant effect as they raise the redox potential and consequently accelerate metal catalysed hydroperoxide decomposition (Waters, 1971).

1.4.7. Dietary Antioxidants

The major source of natural antioxidants is obtained from the diet. These are almost exclusively derived from plants and include carotenoids, flavanoids and vitamins. The most extensively used natural antioxidants are the tocopherols and ascorbic acid. Tocopherols, ascorbic acid, β-carotene and uric acid are of interest as they are capable of participating in the *in vivo* radical defense mechanism. Tocopherols and ascorbic acid mixes are extensively used as a basis for several approaches to the stabilization of oils and foods because of radical exchange reactions between lipid radicals, tocopherol and ascorbic acid.

1.4.7.1. Carotenoids

Carotenoids are pigments widely distributed in nature, accumulating in chloroplasms. They are also found in leaves, flower petals, fruits, roots and also in fungi (Goodwin and Britton, 1988). The carotenoid group include several hundred tetraterpenoid compounds consisting of a sequence of eight isoprene units (Britton, 1988). Their role appears to be in protecting against harmful radiation by reacting with singlet oxygen, thereby preventing photo-oxidation.

1.4.7.2. Flavanoids

Flavanoids are present in the leaf cuticle and epidermal cells in plants and are described as co-pigments, contributing to the colours of flowers, fruits and sometimes leaves. The presence of flavonoids in plant tissues protects them from the damaging effects of UV radiation and is considered essential for the survival of plants.

All these compounds, approximately 4000 in number, have essentially the same 2-phenylchromane structural skeleton and share a common biosynthetic origin.

The flavonoids can be divided into a number of classes dependent upon the degree of oxidation of the central pyran ring, Figure 17:

2-Phenylbenzopyriliums (anthocyanins).

2-Phenylchromones (flavones, flavonols and their dimers; flavanones and dihydroflavonols; isoflavones and isoflavanones).

2-Phenylchromanes (flavans, flan-3-ols, flavan-3,4-diols).

Chalcones and dihydrochalcones.

2-Benzylidene coumaranones ~Aurones.

The antioxidant activities of these compounds have been extensively investigated and their mode of action has been determined (Afanas'ev *et al.*, 1989; Affany *et al.*, 1987; Hart *et al.*, 1990; Mora *et al.*, 1990; Ratty and Das, 1988).

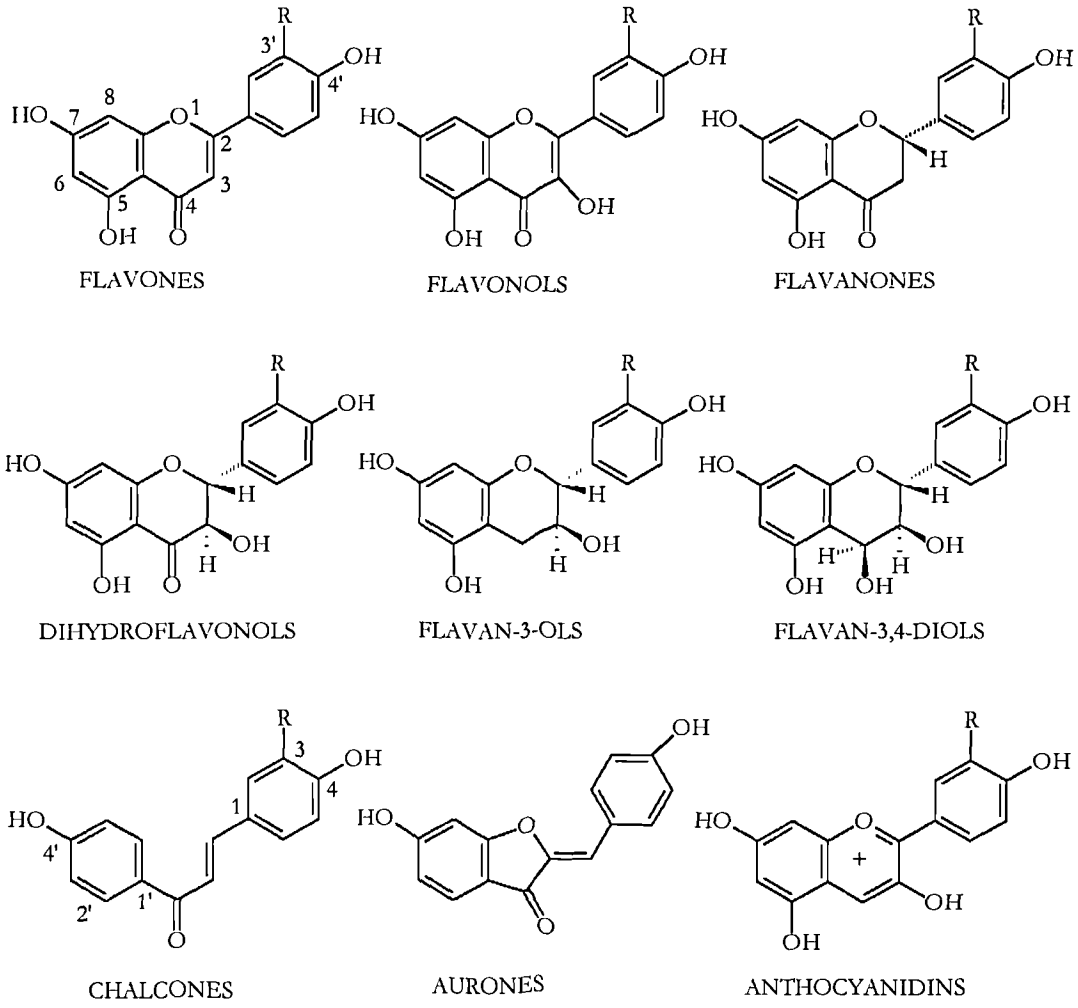


Figure 17. Flavonoids (Bruneton, 1995b).

1.4.8. Vitamins

1.4.8.1. Ascorbic Acid

Ascorbic acid (vitamin C) is a water soluble compound with both antioxidant (Halliwell, 1994) and pro-antioxidant (Wills, 1972; Braughler *et al.*, 1986) properties. It is thought to be essential in the recycling of tocopherol derived radicals generated under oxidative stress.

1.4.8.2. Tocols and the Tocotrienols

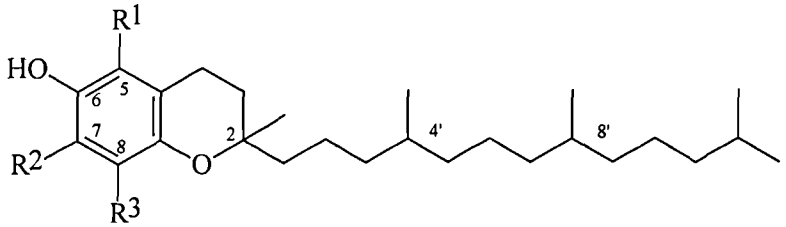
The members of the tocols (Table 6) and the tocotrienols (Table 7) families are designated with α, β, γ and δ, depending upon the number and the position of the methyl groups attached to the chromane ring. The side chain is saturated in the tocols and unsaturated in the tocotrienols.

The tocols are commonly known as tocopherols. There are three centers of symmetry at C-2, C-4' and C-8' in the tocopherol structure. The tocotrienols possess only one center of symmetry, at C-2 in addition to the sites of geometrical isomerism at C-3' and C-7'. Consequently, there are four stereoisomers for each tocopherol and tocotrienol.

1.4.9. Synthetic Antioxidants

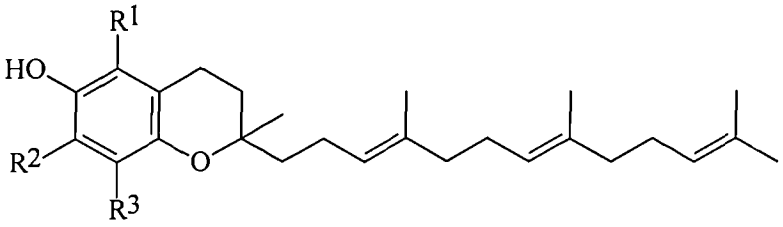
A number of synthetic antioxidants are used in the preservation of foodstuffs and the stabilization of materials such as plastics, rubber, polymers and cosmetics. Their use in food requires the consideration of the technological necessity, the toxicology of lipid peroxidation and antioxidants to be taken into account. Since food additives are subject to the most stringent toxicological testing procedures, only a small number of synthetic compounds are presently used in the food industry.

Table 6. The chemical structure of tocols.

Compound	Formula	Structure
	TOCOLS	
		
Tocol	$C_{26}H_{44}O_2$	R^1:H
	388.64	R^2:H
		R^3:H
8-Methyltocol	$C_{27}H_{46}O_2$	R^1:H
(δ-Tocopherol)	402.67	R^2:H
		R^3:CH_3
5,8-Dimethyltocol	$C_{28}H_{48}O_2$	R^1:CH_3
(β-Tocopherol)	416.69	R^2:H
		R^3:CH_3
7,8-Dimethyltocol	$C_{28}H_{48}O_2$	R^1:H
(γ-Tocopherol)	416.69	R^2:CH_3
		R^3:CH_3
5,7,8-Trimethyltocol	$C_{29}H_{50}O_2$	R^1:CH_3
(α-Tocopherol)	430.72	R^2:CH_3
		R^3:CH_3

Most of the synthetic antioxidants are of the phenolic type, Table 8. Their different capacities are related to their chemical structures and their physical properties such as volatility, solubility, and thermal stability. In addition to the structure - function, there are other considerations determining the use of specific synthetic antioxidants. The type of susceptible product, the nature of the lipids requiring antioxidant protection, its water activity and the storage and manufacturing conditions all govern the efficiency and choice of synthetic antioxidant used.

Table 7. The chemical structure of tocotrienols.

Compound	Formula	Structure
	TOCOTRIENOLS	
		
8-Methyltocotrienol	$C_{27}H_{40}O_2$	R^1:H
(δ-Tocotrienol)	369.62	R^2:H
		R^3:CH_3
5,8-Dimethyltocotrienol	$C_{28}H_{42}O_2$	R^1:CH_3
(β-Tocotrienol)		R^2:H
		R^3:CH_3
7,8-Dimethyltocotrienol	$C_{28}H_{42}O_2$	R^1:H
(γ-Tocotrienol)		R^2:CH_3
		R^3:CH_3
5,7,8-Trimethyltocotrienol	$C_{29}H_{44}O_2$	R^1:CH_3
		R^2:CH_3
(α-Tocotrienol)		R^3:CH_3

The metabolites of synthetic antioxidants may not necessarily be beneficial or safe. BHT, BHA and ethoxyquin can potentially induce several hepatic metabolising enzymes such as epoxide hydroxylase (Williams and Weisburger, 1991), have been linked in animal trails to cancer and liver necrosis (Gutteridge, 1994) and modified tumour induction by certain carcinogens in a number of target organs (Williams and Weisburger, 1991).

1.4.9.1. Butylated Hydroxyanisole and Butylated Hydroxytoluene

Butylated hydroxyanisole (BHA; *tert*-butyl-4-hydroxyanisole) and butylated hydroxytoluene (BHT; 2,6-di-*tert*-butyl-*p*-cresol or 2,6-di-*tert*-butyl-4-methylphenol) account for approximately 75% of commercially sold antioxidants. Both BHA and BHT are simple phenolic compounds with sterically hindered hydroxyl groups, which function mainly as electron or hydrogen donators. Commercial BHA is a

mixture of two isomers, 2-*tert*-butyl-4-hydroxyanisole (2-BHA) and 3-*tert*-butyl-4-hydroxyanisole (3-BHA), and contains 90% 3-isomer. BHT is not as effective as BHA due to the presence of two *tert*-butyl groups, which offer greater steric hindrance than BHA (Sherwin, 1990). Toxicological data for BHA and BHT have recently been reviewed (Madhavi and Salunkhe, 1995).

1.4.9.2. Gallates

Gallates are phenolic compounds with primary or chain breaking properties. Gallates are propyl, octyl or dodecyl esters of gallic acid (3,4,5-trihydroxybenzoic acid). Propyl gallate is the most effective antioxidant and is extensively used by the food industry. Toxicological data for the gallates has recently been reviewed (Madhavi and Salunkhe, 1995).

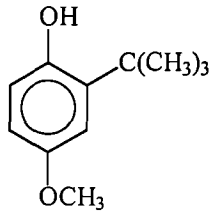
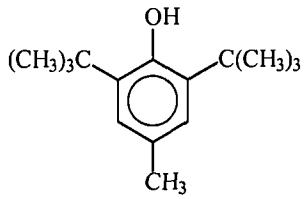
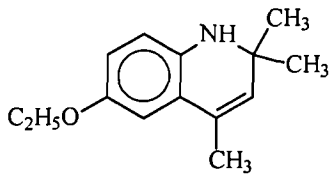
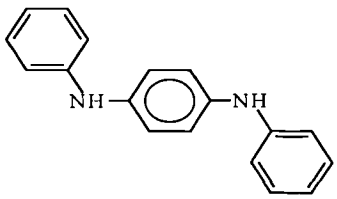
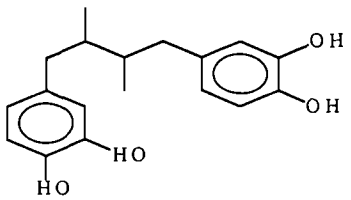
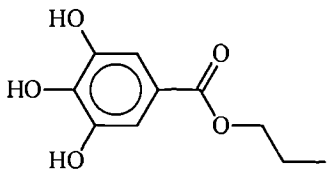
1.4.9.3. Ethoxyquin

Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) acts as a primary or chain breaking antioxidant by readily undergoing oxidation to form a stable free radical ethoxyquin nitroxide. Ethoxyquin nitroxide is more effective as an antioxidant than ethoxyquin, its parent compound (Weil *et al.,* 1968; Lin and Olcott, 1975). Toxicological data for ethoxyquin has recently been reviewed (Madhavi and Salunkhe, 1995).

1.4.9.4. Ethylenediaminetetraacetic Acid (EDTA)

EDTA forms thermodynamically stable complexes with transition metals. The spatial structure of the EDTA anion, which consists of six donor atoms, satisfies the co-ordination number of the most frequently encountered metal ions. Strainless five membered rings are formed on chelation.

Table 8. Antioxidant inhibitors of lipid peroxidation.

 Butylated Hydroxyanisole	Very often added to foodstuffs. Acts as an antioxidant by hydrogen donation, which is very common to all phenolic (and amine) antioxidants.
 Butylated Hydroxytoluene	Very often added to foodstuffs. Non-toxic, but some evidence of metabolism by the liver cytochrome $P\text{-}_{450}$ system. Very large doses cause liver damage in mice.
 Ethoxyquin (Santoquin)	Frequently used in fruit canning. Powerful enzyme inducer *in vivo*. Widely used in longevity experiments in animals.
 N,N'-Diphenyl-p-phenylene diamine (DPPD)	Popular antioxidant *in vitro*. Has been widely used for animal studies of *in vivo* lipid peroxidation.
 Nordihydroguairetic acid (NDGA)	Occurs naturally in resinous exudate of *Larrea divaricata* (American creoaota bush) and in some other plants. Often added to foodstuffs and several polymers (e.g. rubber and lubricants). Binds iron ions.
 Pyropyl gallate	Fairly water soluble. A good inhibitor of lipid peroxidation. Often added to foodstuffs. Binds iron ions.

1.4.10. Enzyme Antioxidants

Prokaryotes and eukaryotes have evolved a number of enzyme systems that prevent the potential damaging effects of harmful radiation and metabolic pro-oxidant stages within cells. The major enzymes are discussed below.

1.4.10.1. Superoxide Dismutase (SOD; EC 1.15.1.1.)

SODs are a family of enzymes with different prosthetic groups possessing the ability to dismutate superoxide radicals. Eukaryotes usually have copper-zinc SOD which is mainly found in the cytosol and manganese SOD which is primarily found in the mitochondria.

Two molecules of superoxide are involved in the dismutation of superoxide radicals. One is reduced while the other is oxidized, resulting in the formation of hydrogen peroxide. As hydrogen peroxide is the end product, efficient removal must be carried out. The effective detoxification of superoxide by SOD, therefore, requires the concerted action of a host of enzymes, namely, catalase and/or glutathione peroxidase.

1.4.10.2. Catalase (EC 1.11.1.6.)

Catalase is a haeme-containing tetrameric protein. The enzyme consists of four identical subunits each containing a haeme (Fe (III)-protoporphyrin) group bound to its active site. Each subunit also contains one molecule of NADPH, which stabilizes the enzyme. Catalase is found in animal and plant tissues in subcellular organelles bound by a single membrane, which are known as peroxisomes.

In animals, catalase is detectable in all organs but especially in the liver and erythrocytes, while the brain, heart and skeletal muscle contain low amounts. Catalase helps in preventing the accumulation of hydrogen peroxide by reducing it to water and oxygen. It has a relatively high capacity for H_2O_2 but a low affinity for it. The dismutation of hydrogen peroxide into oxygen and water has recently been reviewed (Halliwell and Gutteridge, 1989). The reaction process occurs in two steps which can be inhibited by either azide or cyanide (Halliwell and Gutteridge, 1989).

1.4.10.3. Glutathione Peroxidase (GSH-Px; EC 1.11.1.9.)

Glutathione peroxidase was discovered in 1957 as the enzyme which protects against haemoglobin oxidation and erythrocyte haemolysis. The enzyme catalyses the oxidation of reduced glutathione (GSH) to its oxidized form (GSSG) at the expense of hydrogen and organic peroxides to their respective alcohols.

In the oxidized form, two glutathione molecules join as the sulphydryl groups of cysteine are oxidized to form a disulphide (-S-S-) bridge. Selenium in the active site of the reduced GSH-Px is present as selenocysteine (Forstrom *et al.,* 1978; Wendel *et al.,* 1978). GSH reduces selenium then the reduced form of the enzyme reacts with hydrogen peroxide with an approximate rate constant of $5x10^7M^{-1}s^{-1}$ (Halliwell and Gutteridge, 1989). Unlike catalase, glutathione peroxidase has a low capacity for the reduction of hydrogen peroxide, but has a high affinity.

The kinetic mechanism of GSH-Px is a process involving three steps (Flohe *et al.,* 1972; Flohe and Gunzler, 1974). The first involves the oxidation of the enzyme by the peroxide substrate and release of the corresponding alcohol (or water in the case of hydrogen peroxide). Two successive additions of GSH and the release of GSSG follow this. It has been proposed by Ganther *et al.* (1976), that during catalysis the selenium in GSH-Px cycles between selenol (-SeOH) and a selenic acid (-SeH) or between a selenic acid and a selenious acid (-SeOOH). The relatively high levels of GSH present in tissues would probably keep the enzyme in the selenol-selenic acid cycle *in vivo*.

Selenium-dependent, cytosolic, and selenium-independent forms have been identified and characterized. The selenium-dependent form acts preferentially on phospholipid hydroperoxides while the independent form demonstrates a substrate specificity for organic hydroperoxides rather than hydrogen peroxide (Prohaska, 1980).

1.4.10.4. Oxidised Glutathione (GSSG) Reductase

Reduced glutathione is an important cellular component as its oxidation to GSSG is utilized to maintain the oxidative status of cells. Consequently, a high refresh rate of reduced glutathione back from the oxidized form is vital in maintaining the antioxidative balance within cells. GSSG reductase uses reducing

equivalents provided by NADPH to regulate this process when cells undergo oxidative challenge (Ross and Moldeus, 1991).

1.4.10.5. Miscellaneous Enzymes

Radicals such as hydroxyls may react with DNA by both abstraction and addition reactions to produce DNA single strand breaks, altered bases and DNA-protein cross-links. The repair of DNA from such damage may be chemically or enzymatically carried out. The later may involve the action of endo- and exonucleases, glycosylates and ligases. Oxidative damage to proteins maybe repaired by thiol disulphide exchange reactions involving thiol transferases or by rapid and selective intracellular proteolytic enzymes (Ross and Moldeus, 1991).

1.5. Phytochemistry of Plant Volatile Oils

The cells of green plants are the site of a variety of intricate and complex synthetic activities which produce a remarkable selection of organic compounds (Geissman and Crout, 1969). The practical exploitation of many of these is increasing with the application of separation procedures and reliable physical methods of identification and structural determination. The best chromatographic separation techniques (LC, GC, TLC, HPLC) and spectroscopic methods of structure determination (IR, UV, NMR and MS) have led to ever increasing knowledge of the structural types, biosynthesis and ultimately biological significance of plant secondary products. It is likely that many natural products will in time be recognized as possessing important biological activity.

The initial products of photosynthesis are carbohydrates which undergo further metabolic alterations to form simple structured organic molecules of low molecular weights such as carboxylic acids of the citric acid cycle, amino acids which make up the majority of proteins, the common fats and lipids and sugars/sugar derivatives. Primary metabolites are not restricted in their distribution nor characteristic of specific botanical sources but are the synthetic building blocks for specific, genetically controlled enzymatically catalysed reactions which lead to structurally complex secondary metabolites.

Secondary metabolites have a high degree of uniqueness, found in only a single plant species or characteristic of a restricted species, genus or family of plants.

Plants of the same species may contain quite different amounts or representatives of one or another of these classes of compounds. A general trait of these natural products is that few of them at present would appear to possess any essential metabolic function within the plant. Consequently, in terms of DNA, enzymes, photosynthate and energy, their production would appear to be extremely expensive.

1.5.1. Terpenoids

The largest and structurally most diverse class of secondary plant metabolites includes the terpenoids (Geissman and Crout, 1969). Over 20,000 terpenes are known to be produced by plants (Connolly and Hill, 1992). These compounds are synonymously termed terpenoids, terpenes or isoprenoids. The various subgroups are often given the -oid or -ene suffixes interchangeably. They are amongst the chemicals responsible for the medicinal, culinary and fragrant uses of aromatic and plants. Most terpenoids are derived from the condensation of branched five-carbon isoprene units and classified according to the number of these units present in the carbon skeleton. The smallest naturally occurring terpenoids were believed to be derived from two isoprene units and this has lead to the families: monoterpenes (C_{10}) (Figure 18), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes to which the steroids are closely related (C_{30}), tetraterpenes (C_{40}) etc.

In plants, the large molecular weight terpenoids are essential components in metabolism. Pigments such as carotenoids and the phytyl side chain of chlorophyll, sterols of cellular membranes, and plant hormones such as gibberellins and abscisic acid are derived from the terpenoids. A number have been recognized as attractants (Kullenberg and Bergstrom, 1975; Harborne, 1988) and feeding deterrents (Hubbell *et al.,* 1983; Kelsey *et al.,* 1984). They may be involved in allelopathy (Muller, 1966; Rice, 1984); have antibiotic activity (Oh and Sakai, 1967; Piccaglia *et al.,* 1993; Deans *et al.,* 1994a); antifungal activity (Carlton *et al.,* 1992); act as solvents for bioactive lipophilic compounds (Carlton, 1990) and may be mediators in the nutrient cycle (Middleton, 1984).

1.5.1.1. Biosynthesis

The fundamental building block in the biosynthesis of all terpenoids is the five-carbon molecule isoprentenyl pyrophosphate (IPP) which is synthesized from the mevalonic acid pathway, Figure 19. Isoprentenyl pyrophosphate is derived from the sequential condensation of three molecules of acetyl-CoA to produce 3-hydroxyl-3-methylglutarul-CoA. This is followed by a two step reduction to mevalonic acid; sequential phosphorylation to mevalonate-5-phosphate, then mevalonate-5-pyrophosphate and finally a decarboxylative step which generates isoprentenyl pyrophosphate.

A process of isomerisation changes isoprentenyl pyrophosphate to 3,3-dimethylallyl pyrophosphate (DMAPP) a more reactive allylic pyrophosphate isomer which acts as a primer for elongation of the isoprene skeleton by sequential condensations with additional isoprentenyl pyrophosphate units. The enzymes specific for these elongation reactions are collectively known as prenyl transferases. For example, geranyl pyrophosphate (GPP) is generated by geranyl pyrophosphate synthetase and farnesyl pyrophosphate (FPP) is formed by farnesyl synthetase. It is the prenyl pyrophosphates that act as the intermediate precursors of the different terpenoid families. The monoterpenes and the sesquiterpenes are produced from geranyl pyrophosphate and farnesyl pyrophosphate respectively.

Acyclic monoterpenes are derived from geranyl pyrophosphate, through a series of hydrolysis/elimination reactions followed by isomerisation and redox reactions. These monoterpenes are the most abundant monoterpenoids in nature. Cyclic monoterpenes are derived from geranyl pyrophosphate by complex mutli-step reactions carried out by enzymes known as monoterpene cyclases.

These enzymes catalyze the first committed enzymatic steps which lead to the formation of cyclic monoterpenes. Only a small number of cyclic types are generated, including monocyclic and bicyclic structures. Although a single product may be formed by a number of these cyclases, some produce different multiple skeletal types or positional isomers of a single skeletal type. These parent compounds may then undergo further transformation by redox, conjugation and isomerisation reactions to generate the enormous variety of cyclic monoterpenes found in nature. Despite the vast numbers and the structural diversity of these compounds, practically all of them

are derived from one of three pathways: the acetate, mevalonate and shikimate pathways or a combination of two or more of these (Waterman, 1993).

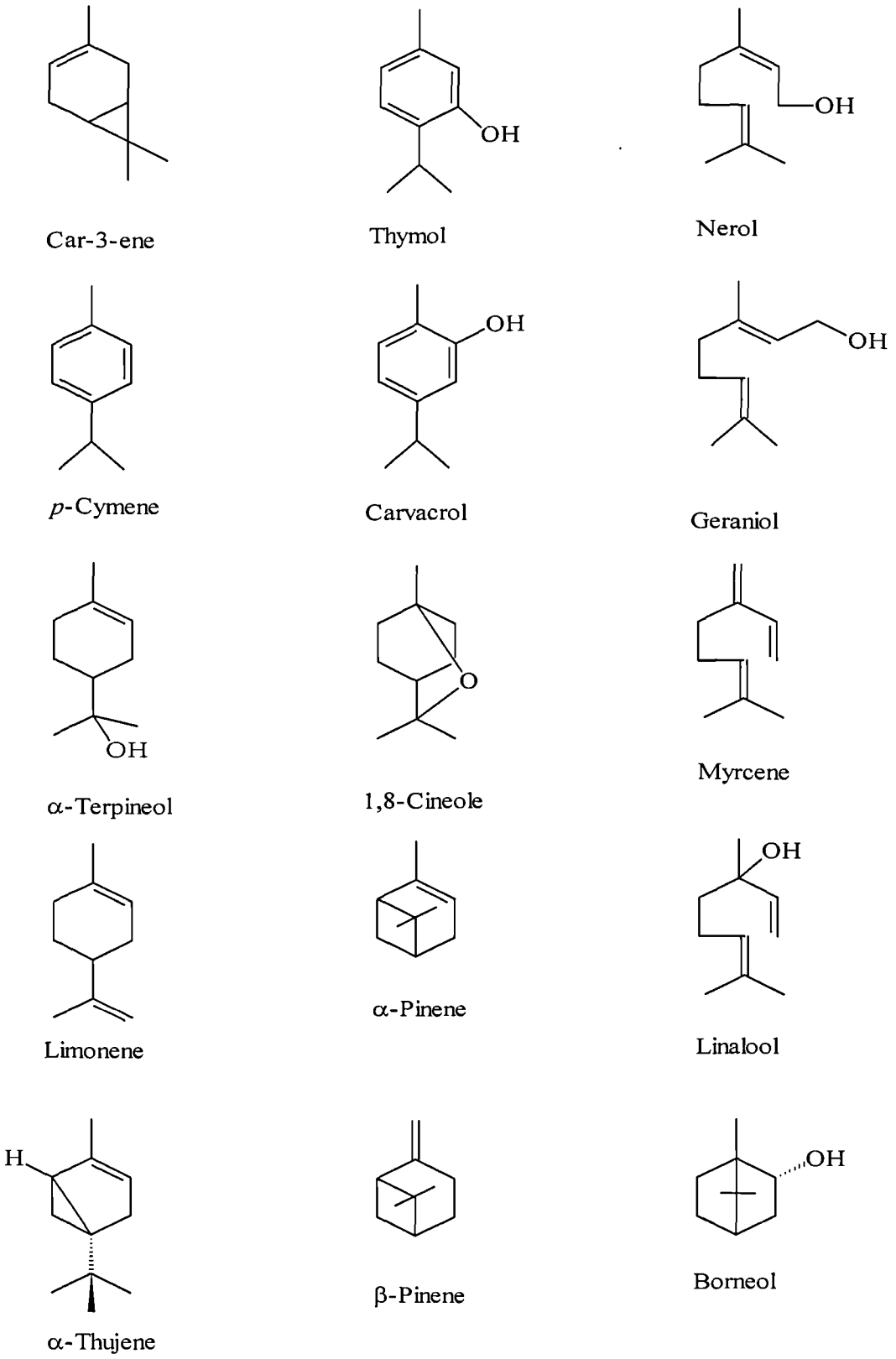


Figure 18. Chemical structures of a number of monoterpenes - C_{10} compounds. (Banthorpe, 1994).

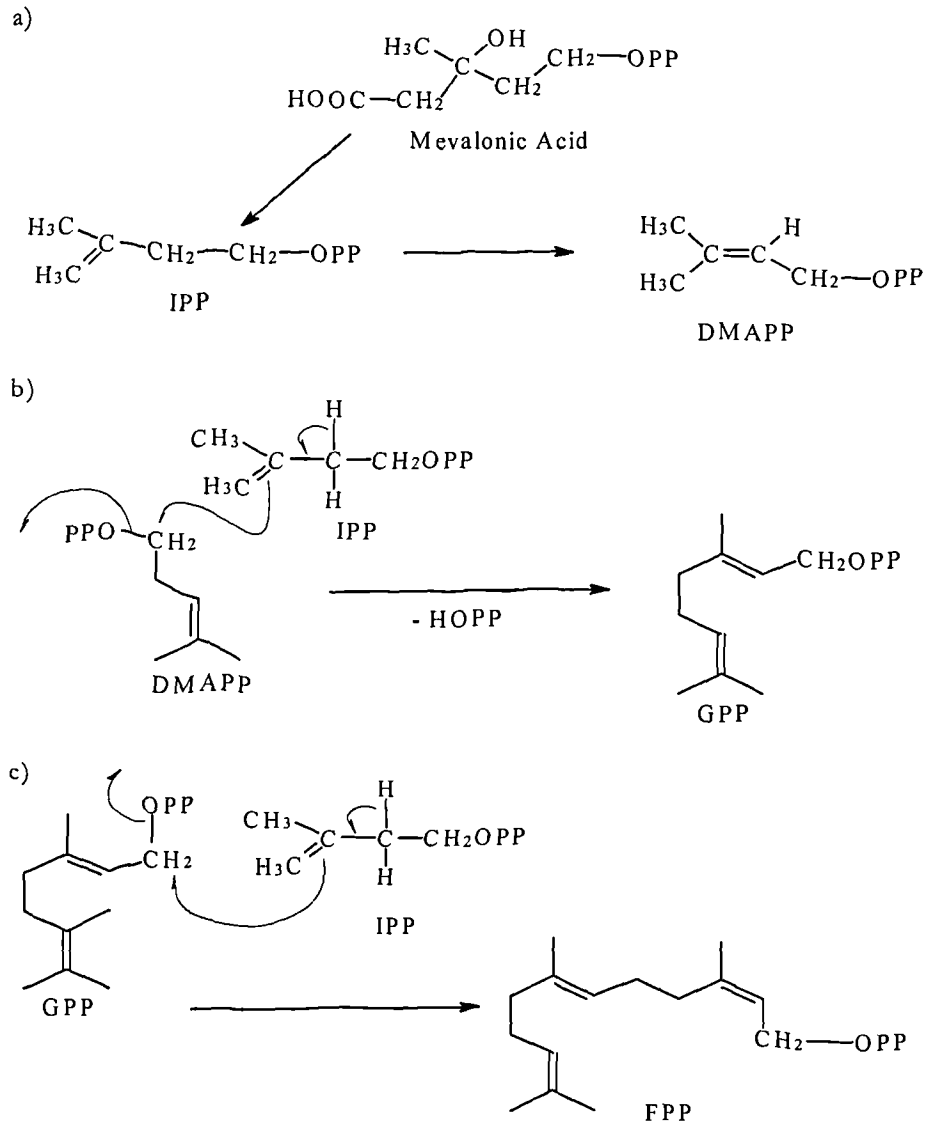


Figure 19. a) Conversion of mevalonic acid to IPP and DMAPP, b) Combination of IPP and DMAPP to give GPP, and c) Combination of GPP with IPP to give FPP. (Waterman, 1993).

Terpenes are wholly generated from the mevalonate pathway. There are numerous reviews and research papers on the synthesis of monoterpenoids and other terpenoids (Hefendehl and Underhill, 1967; Risinger and Hurst, 1968; Geissman and Crout, 1969; Thomas and Bessiere, 1981; Herbert, 1989; Carlton, 1990; Waterman, 1993; Banthorpe, 1994).

1.5.2. Plant Volatile Oils

Plant volatile oils have been described as ***products, generally of rather complex composition, comprising the volatile principles contained in the plants, and more or less modified during the preparation process*** (Bruneton, 1995a); ***products obtained from a plant starting material, either by steam distillation, or by mechanical procedures from the epicarp of citrus fruits, or by simple distillation and is subsequently separated from the aqueous phase by physical methods*** (Bruneton, 1995a); ***the common term used for a volatile oil, normally obtained by distillation, extraction or expression, mostly composed of terpenoids and some benzene derivatives*** (Weiss, 1997a) or ***are the volatile, organic constituents of fragrant plant matter and contribute to both flavour and fragrance. They are extracted either by distillation or by cold pressing (expression). They are not present in all plants*** (Tisserand and Balacs, 1996).

Plant volatile oils are variable mixtures of terpenoids specifically monoterpenes, sesquiterpenes and to a less extent phenylpropenoids - aromatic compounds derived from phenylpropane. Phenylpropenoids are derived from the shikimic acid pathway and are one of the simplest groups of cinnamic acid derivatives, Figure 20. Although they are less abundant, probably less than 50 are known, they are significant components in extracted oils (Waterman, 1993). A number of additional compounds of miscellaneous origin may also be present as a result of steam distillation extraction. These compounds tend to be a variety of low molecular weight aliphatic hydrocarbons (linear, ramified, saturated or unsaturated but rarely specific), acids (C_3 to C_{10}), alcohols (e.g. (3Z)-hexan-1-ol or 1-hexan-3-ol), aldehydes (e.g. octenal, decanal or (2E)-hexanal), acyclic esters or lactones and exceptionally nitrogen or sulphur containing compounds, diterpenes, coumarins and homologues of phenylpropanoids may also be detected.

1.5.2.1. Factors influencing the Yield and Composition of Plant Volatile Oils

A variety of factors affect the yield and composition of volatile oils. These include genetics, morphogenesis, environment (light intensity/duration and altitude) and agricultural practices.

1.5.2.1.1. Genetics

Origin effects occur in plants not only from the same species but from those harvested from different sites. These differences have been discovered in the cases of *Origanum* (Putievsky *et al.,* 1988a; Ozguven and Stahl-Biskup, 1989) and *Thymus vulgarus* (Gouyon *et al.*, 1986). Variation not only can occur in nature, but may also be produced artificially. Genotype and subsequent chemotype variations change the content and composition of volatile oils as demonstrated by Putievsky *et al.* (1984) in *Origanum* plants.

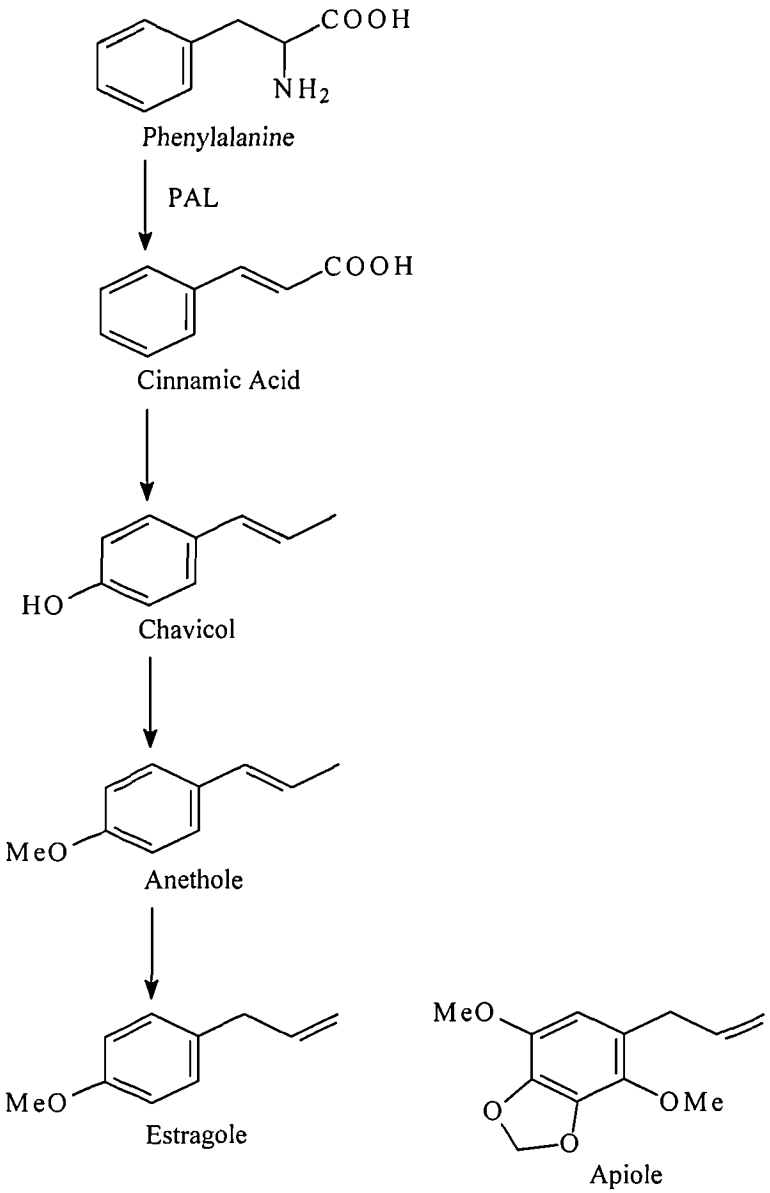


Figure 20. Biosynthesis of phenylpropenes. PAL: Phenylalanine Ammonia Lyase. (Waterman, 1993).

1.5.2.1.2. Morphogenesis

The content and the composition of volatile oils are related to the leaf position and age in shrub plants, especially in the Labiatae family. The younger the leaves, the higher the content and amount of volatile oil per unit of leaf when compared with older leaves (Schratz and Horster, 1970; Lincoln and Langenheim, 1978; Ravid and Putievsky, 1984). In addition, the flowering stage may alter the volatile oil content and composition (Maffei *et al.,* 1986). In some species, a decrease in volatile oil content has been reported during flowering (Ravid and Putievsky, 1984; Dudai *et al.,* 1987) while in others such as in the case of *Origanum*, the content has been reported to increase (Putievsky *et al.,* 1988).

1.5.2.1.3. Environment

Environmental conditions may have a significant effect upon volatile oils (Kastner, 1968; Schratz and Horster, 1970; Weiss and Fluck, 1970). Large variation in the main components may occur in the same plant variety when grown at different locations. The most important of these variables include temperature, day length and light intensity.

1.5.2.1.4. Agricultural Practices

A number of reviews have reported on the effect nutrition has upon the yield and composition of aromatic and medicinal plants (Ruminska, 1978; Franz, 1983). However, the underlying mechanisms involved have not been elucidated. Other practices affecting yield and composition include irrigation, plant growth regulators, micropropagation, harvesting and distillation (Menary, 1994).

1.5.2.2. Oil Isolation Methods

Methods of isolation and production of plant volatile oils have been modernized recently, with continuous distillation and extractions being introduced over the last decade (Boelens, 1997). Methods include the expression of citrus fruits, steam distillation of Labiatae oils, hydrodistillation of flower oils, hydrodiffusion of leaf oils, solvent extraction and supercritical carbon dioxide extraction of flower concretes.

1.5.2.2.1. Expression

For the expression of oils from citrus fruits, there are four main methods: the Italian pellatrice method, the sfumatrice method, the American Brown oil extraction and the FMC Corporation process. Expressed oils always contain a non-volatile residue which can vary in concentration from 2% to 7% (Boelens, 1997).

Pellatrice Method

The pellatrice expression of citrus fruits involves the abrasion of the surface of the entire fruit. The fruits are rotated against an abrasive surface of an Archimedes' screw, which causes oil cells to burst. The released oil is then washed away with a water spray (Shaw, 1979). The oil in the resultant oil-water emulsion is then isolated using centrifugal separators. The benefits of this method are the yields and quality of oil that contains more oxygen containing compounds even though the oil is darker and contains a residue.

Sfumatrice Method

In this method, the peel and pulp from the fruits are removed before treatment and the peel hardened in a lime bath. The oil is isolated from the peel by a ribbed roller pressing and a water spray (Shaw, 1979). The oil is then separated by centrifugation. The benefits of this method include pulp separation, a lighter product and less residue although there is no optimal yield (Boelens, 1997).

Brown Oil Extractor Method

In this method, the whole fruit is used. The fruits move along a bed of rollers covered with needles and a water spray removes the oil-water emulsion. The oil is recovered from the obtained emulsion by centrifugation. The benefits of this method include low solid content and water recycling.

FMC Corporation Method

Over 50% of all citrus oil are isolated by this process (Boelens, 1997). This method is based on the whole fruit extraction principle. The oil is recovered during the juice extraction (Flores and Segredo, 1996) and finally isolated by centrifugation. The advantages of this method include fully automatic process and juice and oil production.

1.5.2.2.2. Distillation

An important method for the isolation of volatile oils is distillation, which include steam distillation, hydrodistillation and hydrodiffusion methods and have been described (Denny, 1988).

Steam Distillation

The volatile oil is extracted from plant material by direct steam, produced in a still or by indirect steam. The still often has a grill at the bottom with the plant material placed into a perforated basket. This method of extraction is used for the production of Labiatae leaf and flower oils, laurel leaf oil, eucalyptus leaf, bitter orange leaf oil and umbelliferous fruit oils, etc. With the exception of the expression of citrus oils, this method is used in extraction of the bulk of volatile oils.

This is the method used routinely in this laboratory for the extraction of volatile oils using a 'Quick Fit' Steam Distillation Apparatus, as specified in the European Pharmacopoeia (Lou, 1980) and as described in British Standard Methods (1984).

Hydrodistillation

Hydrodistillation is mostly carried out with flowers, e.g. bitter orange flower, rose and jasmine. Material is placed in a perforated basket and heated in 2-3 times their weight of water with indirect steam. A volume of water to the weight of the material is distilled. Yield of the separated oils is in general below 0.1% and the distillate water is saturated with the more soluble oxygenated derivatives (Boelens, 1997).

Hydrodiffusion

Hydrodiffusion is carried out with low-pressure steam (<0.1 bar) replacing volatiles from the intact plant material by osmotic action. In the hydrodiffusor, the low-pressure steam flow goes from the top through the vegetable load down to the condenser at the bottom. The isolation sequence of the volatile components is determined to a greater extent by their water solubility. Consequently, the condensate water is saturated with the polar constituents of the oil (Boelens *et al.*, 1990).

1.5.2.2.3. Extraction

Another method for the isolation of volatile oils is the extraction of plant material using solvent extraction, subcritical liquid carbon dioxide and supercritical fluid carbon dioxide extraction.

Solvent Extraction

Solvent extraction can be carried out either by percolation or by immersion. In the percolation method, the solvent runs through the plant material; in the immersion method, the solvent covers the plant material completely. Although hexane is the most often utilized, wide range of solvents including alkanes, haloalkanes, benzenoids, ethers, ketones, etc are used.

Subcritical Liquid Carbon Dioxide

Subcritical liquid carbon dioxide extraction is carried out at 50 to 80 bar and at a temperature of between 0°C to 10°C (Moyler, 1992). Approximately fifty volatile oils obtained by liquid carbon dioxide extraction are commercially available.

Supercritical Fluid Carbon Dioxide Extraction

Supercritical fluid carbon dioxide extraction is carried out at pressures in excess of 80 bar and generally with temperatures above room temperature (Moyler, 1992; Simpson, 1995). Superficial fluid extraction (SFE) is more common method of volatile oil isolation. One may also make a solvent extraction, e.g. hexane, to prepare a concrete and subsequently carry out a SFE-extraction.

1.5.2.3. Quality

The quality control of volatile oils ultimately depends upon the intended use of the oil and therefore, the composition and uniformity of oil. In order to determine the quality the following should be determined (Waterman, 1993):

1. Botanical confirmation of identity and quality of the source(s).
2. Assessment of the physical characteristics of the oil.
3. Measurement of the concentration of a particular component(s) in the oil.
4. The qualitative or quantitative analysis of the oil composition, using chromatographic/spectroscopic and perhaps sensory analysis.

1.5.2.3.1. Identity and Quality of Volatile Oils

This aspect of quality control involves the botanical (morphological and anatomical) examination of the plant material to ensure adulteration with other plant species has not occurred; ensuring that plant material is appropriately stored prior to oil extraction in order to prevent oil loss and contamination with fungi and other pests and carefully monitored extraction to prevent over 'cooking' and ensure reproducibility.

1.5.2.3.2. Physiochemical Standards

Physiochemical standards often comprise acid, alcohol, carbonyl and ester number; the solubility in ethanol/water at various concentrations; specific gravity, optical rotation, refractive index, freezing/congealing, flash point, moisture content and evaporative residue analyses. The standard specifications for specific volatile oils may be obtained from a variety of sources including the International Organisation of Fragrance Association (IFRA), the International Organisation for Standardization (ISO), the International Organisation of Flavour Industries (IOFI), the International Federation of Essential Oils and Aroma Trades (IFEAT), the Essential Oil Association of the United States (EOA), in the Food Chemicals Codex (1996-IV), Monographs of the Research Institute Fragrance Materials (RIFM), the pharmacopoeias (e.g. EP, BP, USP, DAB, etc), in published country standards (e.g. AFNOR) and a variety of publications including the *Journal of Essential Oil Research*, *Flavour and Fragrance Journal* and the *Perfumer and Flavorist* journal.

1.5.2.3.3. Chromatographic Analysis

Gas chromatography (GC) is a useful quantitative and qualitative technique for the determination of the chemical composition of volatile oils. The volatile oil is volatilized in the injection port of the instrument and entrained with the mobile phase (usually either helium, hydrogen or nitrogen) before passing through a high resolution, high precision fused silica capillary column packed with a stationary liquid phase (e.g. DB-5). By using various stationary liquid phases of different physiochemical properties, oil components are capable of being partitioned and then quantitatively detected, usually by using a flame ionization detector (FID).

Providing that an volatile oil is run through a GC instrument under the same conditions (i.e. type of column, mobile phase flow rate, column temperature and

temperature ramps) the oil's constituents should be eluted at the same time upon each run. From the retention times of these compounds, the identity of each component may be determined on subsequent analyses. However, in practice, it is unlikely that run conditions are identical especially over the life-time of the column, consequently an internal standard is used (ideally two – one which elutes at the beginning of the run and a second which elutes in the second half of the run) to account for possible variations in retention times. In practice, component identification is augmented by the co-elution of authentic components, the comparison with the Kováts' Retention Index and other retention indices (e.g. relative to C_8-C_{24} *n*-alkanes on a DB-1 column, Baratta *et al.*, 1998) either corrected (less the time for elution of an unretained component) or not corrected as a reference.

1.5.2.3.4. Spectroscopic Analysis

Often GC analysis is insufficient to identify all the components present in an volatile oil. In such cases, spectroscopic methods must be utilized. These may include mass spectroscopy (MS), infrared spectroscopy (IR) and $^{1}H/^{13}C$-nuclear magnetic resonance (NMR) techniques. GC separation interfaced with MS is a useful technique for the identification of unusual or minor/trace components.

1.5.2.4. Biological Activities

The biological activities of volatile oils have been recognized since antiquity (Deans and Waterman, 1993). Amongst these properties, antibacterial (Osawa *et al.*, 1990; Piccaglia *et al.*, 1993; Lattaoui and Tantaoui-Elaraki, 1994; Baratta *et al.*, 1998a; Baratta *et al.*, 1998b; Baratta *et al.*, 1998c), antifungal (Pauli and Knobloch, 1987; Tantaouielaraki and Errifi, 1994; Viollon and Chaumont, 1994; Paster *et al.*, 1995; Baratta *et al.*, 1998a; Baratta *et al.*, 1998b; Baratta *et al.*, 1998c) and antioxidant (Lagouri *et al.*, 1993; Dorman *et al.*, 1995a; Dorman *et al.*, 1995b; Ozcan, 1995; Schwarz *et al.*, 1996; Baratta *et al.*, 1998a; Baratta *et al.*, 1998b; Baratta *et al.*, 1998c) are the chief bioactivities of interest to man, Table 9.

Pharmacological properties include spasmolytic, sedative and irritating properties. *In vitro*, plant volatile oils such as angelica, sweet basil, chamomile, clove, balm, mint and thyme have marked spasmolytic activity on guinea pig ileum and in fewer cases (anise and sweet fennel) increase the phasic contractions of this organ (Bruneton, 1995a). When used externally, increased capillary blood flow,

substantial rubefaction, a sensation of heat and slight local anesthetic activity are effects noted for plant volatile oils, which many ointments, creams or gels utilize to relieve sprains, soreness, strains and other joint or muscular pains. Oils such as eucalyptus, pine or niaouli stimulate mucus cells, increase the mobility of the ciliated epithelium in the bronchia while juniper is thought to enhance the renal excretion of water by a direct local effect (Bruneton, 1995a). A variety of sources are available which detail numerous historical and experimentally investigated properties for aromatic and medicinal plants. These include: Grieve's A Modern Herbal: The Medicinal, Culinary, Cosmetic and Economic Properties, Cultivation and Folklore of Herbs, Grasses, Fungi, Shrubs and Trees with All their Modern Scientific Uses; Bentley's and Trimen's Medicinal Plants; Clarke's Dictionary of Materia Medica; Potters' Cyclopedia of Botanical Drugs and Preparations; Stephenson's and Churchill's Medical Botany and the English and French official Pharmacopoeias, amongst other journals.

When discussing their bioactivities, a number of points should be considered. The activity of an volatile oil should not be confused with that of the plant from which it came from, e.g. rosemary volatile oil is an antibacterial agent whereas rosemary (plant) infusion traditionally used to treat miscellaneous digestive symptom is based upon antispasmodic and choleretic properties. If it is possible to study and ascribe biological or pharmacological effects of pure monoterpenes, sesquiterpenes or phenylpropenoids identified an oils constituent, it is not necessarily possible to discuss the pharmacology, pharmacokinetics or metabolism of a volatile oil as an oil is mixture of constituents. Finally, the properties attributed to volatile oils *per se* are too broad to permit generalizations, as this would be overly simplistic.

Table 9. Examples of commercially significant aromatic and fragrance source plants and their useful secondary metabolite.

Adapted from Leung, 1980; Tyler *et al.*, 1981 and Windholz *et al.*, 1983.

Family/Botanical Name	Common Name	Principal/Useful Metabolite(s)	Uses/Therapeutic Category
Annonaceae			
Cananga odorata Hook. f.	Ylang-ylang	Geraniol and linalool	Perfumery.
Burseraceae			
Bursera delpechiana Pois. ex Engl. Mexico.	Linaloe	Linalool	Perfumery.
Compositae (Asteraceae)			
Artemisia absinthium L.	Wormwood; absinthe	Thujyl alcohol	Flavouring; anthelmintic; antimalarial.
A. dracunculus L.	Tarragon	Methyl chavicol (estragole)	Flavouring; perfumery.
Geraniaceae			
Pelargonium odoratissimum Ait.	Geranium	Geraniol esters	Perfumery.
Gramineae (Poaceae)			
Cymbopogon (*Andropogon*) *citratus* (DC.) Stapf.	Lemon grass	Citral	Perfumery; source of citral.
C. (*Andropogon*) *nardus* (L.)	Citronella	Geraniol, *d*-citronellal	Perfumery; insect repellent.
Labiatae (Lamiaceae)			
Hedeoma pulegiodes (L.) Pers.	American Pennyroyal	Pulegone	Aromatic carminative.
Lavandula officinalis Chaix. (*L. augustifolia*)	Lavander	Linalyl acetate	Perfumery; flavour; carminative.
Melissa officinalis L.	Balm; Lemon Balm	Citral	Seasoning.
Ocimum basilicum L.	Sweet Basal	Methyl chavicol	Flavouring; perfumery.
Origanum marjorana L.	Marjoram	Terpinene	Perfumery.

Table 9 continued. Examples of commercially significant aromatic and fragrance source plants and their useful secondary metabolite.

Adapted from Leung, 1980; Tyler *et al.*, 1981 and Windholz *et al.*, 1983.

Family/Botanical Name	Common Name	Principal/Useful Metabolite(s)	Uses/Therapeutic Category
O. vulgare L.	Oregano; wild marjoram	Carvacrol	Perfumery.
Salvia offinialis L.	Sage	Cineole	Flavouring; antisecretory.
Thymus vulgaris L.	Thyme	Thymol; carvacrol	Rubefacient; antiseptic; carminative; flavouring.
Lauraceae			
Cinnamomum camphora L.	Camphor	*d*-Camphor	Industrial Solvent; perfumery; rubefacient; insect repellent.
Sassafras albidum (Nutt.) Nees	Sassafras	Safrole	Fragrance; topical anti-infective; ediculicide; carminative.
Myristicaceae			
Myristica fragrans Houtt. (*M. aromatica*)	Nutmeg	*d*-Camphene; safrole; myristicin	Flavour; carminative.
Myrtaceae			
Eucalyptus dives Schauer.	Eucalyptus	Eucalyptol (cineole)	Inhalation expectorant; anthelminthic; local antiseptic.
Myrtus communis L.	Myrtle	α-pinene, eucalyptol	-
Pimenta (*Myrica*) *acris* (Sw.) Kostel.	Bay	Eugenol	Carminative; flavour.
Oleaceae			
Jasminum officinale L.	Jasmine	Jasmone	Perfumery.
Pinaceae			
Abies sibirica Ledeb.	Siberian fir	Bornyl acetate	Flavour; perfumery; expectorant.
Juniperus communis L.	Juniper	Pinenes	Flavour (liqueurs); perfumery; diuretic.

Table 9 continued. Examples of commercially significant aromatic and fragrance source plants and their useful secondary metabolite.

Adapted from Leung, 1980; Tyler *et al.*, 1981 and Windholz *et al.*, 1983.

Family/Botanical Name	Common Name	Principal/Useful Metabolite(s)	Uses/Therapeutic Category
Rubiaceae			
Coffea arabica L.	Coffee	Caffeine	Coffee; stimulant.
Rutaceae			
Citrus aurantium L.	Orange (neroli)	Limonene, *l*-linalool	Perfumery; flavour.
C. aurantium var. *bergamia* Risso.	Bergamot	*l*-Linalyl acetate	Perfumery; fragrance.
Santalaceae			
Santalum album L.	Sandalwood (Santal)	Santalols	Urinary anti-infective; perfumery; cosmetics.
Umbelliferae			
Carum carvi L.	Caraway	*d*-Carvone, *d*-limonene	Flavouring; perfumery; carminative.
Corianderum sativum L.	Coriander	*d*-Linalool	Flavouring; perfumery; carminative.
Petroselinum sativum S. Wats.	Parsley	Apiole	Flavouring.
Foeniculum vulgare Mill.	Fennel	Anethole	Flavour; carminative.
Valerianaceae			
Valeriana officinalis L.	Valerian	Bornyl esters	Sedative.
Zingiberaceae			
Curcuma domestica Loir. (*C. longa* L.)	Turmeric, turmeric	Curcumin	Condiment; colouring material (dye).

1.5.3. Volatile Oils of Interest: Their Composition and Potential Properties

1.5.3.1. Black Pepper *(Piper nigrum)*

Synonyms: Piper.
Family: Piperaceae.

1.5.3.1.1. Botany

The genus *Piper* belongs to the family Piperaceae which includes over 700 species, distributed in both hemispheres (Parmar *et al.*, 1997). Pepper is native to wet tropical forests with its center of origin and diversity at the West Ghats, Tamil Nadu State. It is also wild in Assam and Borneo and has been introduced wherever it would possibly grow. The ripened fruit of black pepper is the source of white pepper, while the unripe fruit of the same species is the source of black pepper. It is a perennial woody climber reaching to 10m. Under cultivation, mature vines have a bushy, columnar shape. Climbing stems are tinged green with purple when young which turn dark green with age. The leaves are usually dark shiny green above, paler and glandular below. The leaves also contain a volatile oil which differs from the berry oil, with a sent resembling lime.

1.5.3.1.2. Volatile Oil

The steam-distilled oil is composed principally of monoterpenoid hydrocarbons and smaller amounts of sesquiterpenoid hydrocarbons, with oxygenated compounds being relatively minor components (Weiss, 1997). Constituents have been reported as including 15 monoterpenoids, 21 sesquiterpenoids, 32 oxygenated monoterpenoids, 4 phenyl esters, 12 oxygenated sesquiterpenoids and 20 miscellaneous compounds (Pino, 1990). The monoterpenoid fraction shows the greatest variation (% composition range) [limonene 0-40%; β-pinene 5-35%; α-pinene 1-19%; α-phellandrene 1-27%; β-phellandrene 0-19%; sabinene 0-20% and δ-3-carene tr.-10%] with minor differences occurring in the sesquiterpenoid and oxygenated compounds [β-caryophyllene 9-33%; α-humulene 2-6%, α-bergamotene 1-4%] (Weiss, 1997). Typically, the representative composition (% composition range) of the volatile oil of black pepper includes the monoterpenes

α-pinene 2-9%, β-pinene 5-14%, α-thujene 0.5-3.5%, sabinene 9-19%, α-terpinene 0.4-2.8%, δ-3-carene 1-15%, myrcene 1.6-2.5%, limonene 17%, α-phellandrene 5-9%, δ-elemene 2.6%, *p*-cymene 1-2.8%, γ-terpinene 0.5-3.9%, terpinolene 0.5-1.5% and camphene; the sesquiterpenes β-caryophyllene 9-29%, α-humulene 1-2%, α-guaiene, α- and β-cubebene 0.2-1.6%, α- and β-selinenes 0.5-7.7%, α- and β-elemene 0.3-2.4%, β-bisabolol 2-5%, calamenene, α-copaene 0.5-1.5%, β-farnesene 1-3%, zingiberene (trace), bergamotene 0.5%, ar-curcumene 0.5%; the alcohols terpinen-4-ol <1%, α-terpineol 0.1%, linalool <1%, *trans*-pinocarveol, *trans*-carveol, elemol 0.5% and α-bisabolol 0.1%; the aldehyde piperonal; the ketones di-hydrocarvone 0.05% and piperitone <1% and the oxide caryophyllene oxide 0.6%.

1.5.3.1.3. Bioactivities

Various extracts from *Piper* species have been reputed in the Indian Ayurvedic and the folklore medicines of Latin America and the West Indies to possess a variety of medicinal properties. It has been reported that these include stimulatory, carminatives and febrifuge properties (Grieve, 1994). It has been used to aid digestion, atonic dyspepsia and turbid conditions of the stomach (Grieve, 1994). The activities of the volatile oil of black pepper include antibacterial (Pérez and Anesini, 1994), anticarcinogenic, antifungal, antihepatotoxic (Koul and Kapil, 1993; Singh and Roa, 1993a), anti-inflammatory (Mujumdar *et al.*, 1990a), anti-irritant, metabolic (Mujumdar *et al.*, 1990b) and antioxidant (Nakatani *et al.*, 1986) properties.

1.5.3.2. Clove (*Syzygium caryophyllus* Gaertn.)

Synonyms: *Syzygium aromaticum* (L.) Merr. & Perry; *Eugenia aromatica* Kuntze; *E. caryophyllata* Thunb.; *E. caryophyllus* (Sprengel) Bull. & Harr.; *Caryophyllus aromaticus* L.

Family: Myrtaceae.

Genus: *Syzygium* Gaertn.

1.5.3.2.1. Botany

The clove tree was long known as *Eugenia aromatica* or *E. caryophyllata*, but a revision of the larger *Eugenia* genus resulted in clove being reclassified into the genus *Syzygium* Gaertn. (Schmid, 1972). The tree clove grows naturally in a tropical maritime climate, Madagascar, Zanzibar or Comoro, although plant material also

imported from the East and West Indies, Mauritius and Brazil. Cultivated clove is an evergreen highly aromatic tree which grows to 15m, generally in a pyramidal manor when young, cylindrical when old, bearing glossy green leaves, fragrant red flowers and purple fruit. Clove trees are primarily cultivated to produce clove the spice while clove oil production is a complementary activity.

1.5.3.2.2. Volatile Oil

The volatile oil from clove can be extracted by steam or water distillation from either clove buds, leaves or stem. The representative composition (% composition range) of the volatile oil of clove includes the monoterpene pinene; the sesquiterpenes α- and β-caryophyllene 5-13%, α- and β-humulene 0.5-1.5%, α-cubebene 0.01-0.3%, α-copaene 0.1-0.2%, calamenene 0.2-0.5%; the phenols (60-90%) eugenol 36-85%, isoeugenol 0.1-0.25%, acetoeugenyl 11-21%; the esters (20-25%) eugenyl acetate 0.5-12%, 2-nonanyl acetate (trace), α-terpinyl acetate 0.1-0.2%, benzyl acetate (trace), methyl-benzoate 0.04-0.13% and the oxides humulene epoxide (trace) and caryophyllene oxide trace-1.8% (Weiss, 1997).

1.5.3.2.3. Bioactivities

Medicinal action and uses of clove include stimulating and carminative properties and has been reportedly used in the treatment of nausea, flatulence, indigestion and dyspepsia. As a local irritant, it has been reported to stimulate peristalsis. It is a strong germicide, a powerful antiseptic, a mild local anesthetic and stimulating expectorant in phthisis and bronchial troubles (Grieve, 1994). The volatile oils extracted from clove demonstrate a number of important biological properties including antibacterial (Briozzo *et al.*, 1989; Aureli *et al.*, 1992; Cai and Wu, 1996; Ouattara *et al.,* 1997), anticarcinogenic (Zheng *et al.,* 1992) antifungal (Llewellyn *et al.,* 1981; Hasan and Mahmoud, 1993; El-Maraghy, 1995; Tombe *et al.,* 1995; Martini *et al.,* 1996), antioxidant (Oya *et al.*, 1997), and pharmacological (Briozzo *et al.,* 1989; Kumari, 1991; Saenz *et al.,* 1996) properties.

1.5.3.3. Geranium (*Pelargonium graveolens* L'Herit)

Synonyms: *Geranium radula* Roth., P. intermedium Knuth.
Family: Geraniaceae.

1.5.3.3.1. Botany

Pelargonium is a small much branched perennial shrub to 1.3 m but generally smaller, which forms dense spreading bush. The leaves are fragrant, on stems and branches, opposite, 6.5-13.5 cm by 6-12 cm, villous, highly divided, with five to seven palmate lobes. Species are a dull green. The fruit is long and pointed and although pelargoniums do not normally produce seeds, cultivars seed readily.

1.5.3.3.2. Volatile Oil

The representative composition (% composition range) of the volatile oil of geranium includes the monoterpenes α-phellandrene (trace), β-phellandrene, α-pinene 1%, β-pinene 0.2%, myrcene 0.2%, limonene 0.2%, *cis*-ocimene 0.2%; the sesqiterpenes guaia-6,9-diene 3.9-5.3%, guaiazulene, α-copaene, δ-cadinene, γ-cadinene, α-bourbonene, β-caryophyllene 0.7%; the alcohols citronellol 21-45%, geraniol 17-25%, linalool 1-13%, nerol 1.2%, α-terpineol 0.7% (*monoterpenols*), 10-epi-γ-eudesmol 1% (*sesquiterpenols*) and phenyl ethyl alcohol <1% (*aromatic*); the aldehydes (bourbon variety) neral, geranial 0-9% and citronellal 0-1%; the ketones menthone 0.6-3%, isomenthone 4-8.4%, piperitone, methyl-heptanone and furo-pelargone 0.4% and the oxides (Chinese variety) *cis*-rose oxide 2-25%, *trans*-rose oxide 1%, *cis*-linalool oxide 0.6% and *trans*-linalool oxide 0.2% (Weiss, 1997).

1.5.3.3.3. Bioactivities

Geranium has been reported to be useful as an astringent and tonic, in the treatment of diarrhoea, cholera and chronic dysentery (Grieve, 1994) The volatile oil of geranium has been attributed a number of biological properties including antibacterial (Ivancheva *et al.,* 1992; Pattnaik *et al.,* 1996), antifungal (Pattnaik *et al.,* 1996), antiviral (Zgorniak-Nowosielska *et al.,* 1989; Ivancheva *et al.,* 1992; Serkedjieva, 1995), antioxidative (Fukaya *et al.,* 1988) and pharmacological (Petkov *et al.,* 1974; Manolov *et al.,* 1977) properties.

1.5.3.4. Lovage (*Levisticum officinalis* L.)

Synonyms: *Ligusticum Levisticum* L.; Old English Lovage; Italian Lovage; Cornish Lovage.

Family: Umbelliferae.

1.5.3.4.1. Botany

Lovage is a stout, umbelliferous, strong growing plant reaching 2 meters in height, belonging to the family Umbelliferae. Originally, *Levisticum officinalis* L. was closely related to *Levisticum persicum*, which grows in the hills of southern Iran and South West Europe (Tutin, 1968). It has thick and fleshy roots, 12 or 15 cm long which are shaped like a carrot, greyish brown on the outside. The stems divide towards the top to form opposite whorled branches which in June and July bear umbels of yellow flowers. These are followed by small aromatic fruit, brownish-yellow and ovoid or oblong in colour and shape. The odour of the whole plant is very strong.

1.5.3.4.2. Bioactivities

Lovage was much used as a drug plant in as early as the fourteenth century, its medicinal reputation probably founded upon its pleasing aromatic odour (Grieves, 1994). It's uses have been reported to include the treatment of cancer and fever and to posses abortifacient, antiseptic, carminative, expectorant and stimulant properties. Members of the Umbelliferae family are known to contain furocoumarins (Nielsen, 1970) which may pose toxicological risks (Grossweiner, 1984; Knudsen and Kroon, 1988; Epe *et al.,* 1993; Lagey *et al.,* 1995).

1.5.3.5. Melissa (*Melissa officinalis* L.)

Synonyms: Balm.

Family: Labiatae/Lamiaceae.

1.5.3.5.1. Botany

Melissa officinalis L., originally indigenous to Turkey, is a perennial shrub which grows in tufts, often found wild in woods, along edges of country roads, in hedges and other cool places. Its stems are erect which bear opposite leaves with rough, wafer-like surfaces which release a lemony odour when crushed. The flowers

are irregularly shaped, white or inkish and are whorled in axillary groups, spaced far away.

1.5.3.5.2. Volatile Oil

Melissa officinalis L. contains a number of constituents (Hefendehl, 1970; Thieme and Kitze, 1973; Brieskorn and Krause, 1974; Morelli, 1977). The representative composition (% composition range) of the volatile oil of melissa includes the monoterpenes *trans*-ocimene 0.2%, β-bourbonene 0.3% and limonene 0.2%; the sesquiterpenes β-caryophyllene 8-10%, α-copaene 4-5%, β-elemene <1%, α-humulene <1%, δ-cadinene 1% and γ-cadinene 1%; the alcohols linalool 0.4-1.3%, nerol <1%, geraniol <1%, citronellol <1%, isopulegol <1% (*monoterpenols*), α-gadinol 0.3%, elemol <1%, (Z)-3-hexanol 0.1% and 1-octen-3-ol 1.3% (*sesquiterpenols*); the ketones 6-methyl-5-hepten-2-one 4.5% and hexahydrofarnesyl acetone 0.2%; the esters geranyl acetate <0.5% and neryl acetate and citronellyl acetate; the oxides 1,8-cineole <0.5% and caryophyllene oxide 2.5-3.6%; the aldehydes neral 22-24%, geranial 32-37% and citronellal 0.7-2.2%; the coumarin aesculetine and others including 3-octanone 0.6% and methyl-heptanone 0.6%.

1.5.3.5.3. Bioactivities

Traditionally, *Melissa officinalis* L. was been used to treat the symptoms of neurotoxic disorders in adults and children and functional dyspepsia. The volatile oil has been attributed a number of activities including antimicrobial (Larrondo *et al.,* 1995), antiviral (Herrmann and Kucera, 1967; Dimitrova *et al.,* 1993), antioxidant (Lamaison *et al.,* 1990; Lamaison *et al.,* 1991; Dorman *et al.,* 1995a) and pharmacological (Glowatzki, 1970; Chakurski *et al.,* 1981; Chlabicz and Galasinski, 1986; Soulimani *et al.,* 1991) properties.

1.5.3.6. Monarda (*Monarda citriodora* var. *citriodora* Cerv. ex Lag.)

Synonyms: Bergamot
Family: Labiatae/Lamiaceae.

1.5.3.6.1. Botany

Monarda citriodora var. *citriodora* Cerv. ex Lag. belongs to a genus of approximately 15 species of annuals and clump-forming, rhizomatous herbaceous

perennials found growing in dry scrub, prairies and woodlands in North America. Simple or sparsely branching square stems bear alternate, opposite, lance-shaped to oval, usually toothed but sometimes entire, aromatic, mid- to dark green or purple-green leaves with conspicuous veins.

1.5.3.6.2. Volatile Oil

The representative composition (% composition range) of the volatile oil of Monarda from leave material includes α-pinene (0.9-1.4%), β-pinene (0.1-0.7%), myrcene (0.2-2.0%), α-terpinene (1.7-10.0%), limonene (0.5%), 1,8-cineole (0.3-0.6%), γ-terpinene (tr.-3.0%), *p*-cymene (13.0-22.8%), β-caryophyllene (0.6-0.7%), terpinen-4-ol (1.5-2.1%), α-terpineol (tr.-0.5%), geraniol (0-0.4%), carvacrol (3.6-4.3%) and thymol (50.7-56.9%) (Collins *et al.*, 1994; Bishop and Thornton, 1997).

1.5.3.6.3. Bioactivities

Medicinal action and uses of monarda have been reported to include rubefacient, stimulant and carminative properties and used in the treatment of flatulent colic, as a diaphoretic and emmengogue or as a diuretic in urinary disorders (Grieves, 1994).

1.5.3.7. Nutmeg (*Myristica fragrans* Houtt.)

Synonyms: *M. officinalis* L.f.; *M. moschata* Thunb.; *M. aromatica* Swartz; *M. amboinensis* Gand.
Family: Myristicaceae.

1.5.3.7.1. Botany

Nutmeg is a spreading dioecious evergreen tree reaching to 15m with dark green leaves, yellow flowers without petals and large yellowish fruit and belongs to the genius *Myristica* of the family Myristicaceae. Branching is extensive beginning almost at the butt. The twigs are slender, glabrous, greyish-brown and carry a large number of leaves that grow to form a dense canopy. All parts of the tree are aromatic. It usually grows in tropical lowland rain forests from India and Sri Lanka eastwards through South East Asia to Taiwan, the Pacific islands and Australia.

1.5.3.7.2. Volatile Oil

Nutmeg volatile oil has been described as a pale yellow to watery white mobile liquid with a fresh, warm-spicy and aromatic, with a rich sweet-spicy body-note (Wiess, 1997). The main constituents of nutmeg oil have been reported as monoterpene hydrocarbons 61%-88%, oxygenated monoterpenes 5%-15% and aromatic ethers 2%-18% (Weiss, 1997). The representative composition (% composition range) of the volatile oil of nutmeg includes the monoterpenes α-pinene 14-25%, β-pinene 10-15%, myrcene 2%, sabinene 14-35%, α-terpinene 2-4%, γ-terpinene 1.9-7.7%, limonene 3.7-4%, β-phellandrene, camphene <1%, α-phellandrene 0.7-1%, *p*-cymene 1.1-3.1% and terpinolene 0.9-1.7%; the sesquiterpene β-caryophyllene 0-1%; the alcohols terpinen-4-ol 4-8.2% and α-terpineol 0.4-1.2% (*monoterpenols*); the phenolic esters safrole 0.7-1.7%, myristicin 2.9-10.4%, elemicin 0.4-2.1%, eugenol 0.2% and methyl-eugenol 0.6%; the oxide 1,8-Cineole 2-3% and others *trans*-sabinene hydrate <1% and *cis*-sabinene hydrate <1% (Williams, 1996).

p-Cymene, *p*-methyl-isopropenyl benzene, cyclamen aldehyde and cumene have been described as artifacts if present (Weiss, 1997), while traces of myristic acid probably arise from the hydrolysis of its glyceryl esters (Weiss, 1997). Commercial steam distilled oils tend to differ from natural extracts by containing a higher proportion of monoterpenes, owing to incomplete distillation of the oxygenated components. The notable constituents in West Indian oil are camphene, sabinene, α- and β-pinene, myristicin, terpinen-4-ol, γ-terpinene, limonene, myrcene, 1,8-cineole, α-terpinene, *p*-cymene, terpinolene, elemicin, safrole, methyl eugenol, *cis*-piperitol, α-phellandrene, and eugenol. The East Indian oil contains the carcinogens safrole and methyl eugenol and the suspected carcinogen elemicin. Though also found in the West Indian volatile oil, these carcinogens are not present at high concentrations.

1.5.3.7.3. Bioactivities

Medicinal action and uses of nutmeg have been reported to be as a local stimulant to the gastro-intestinal tract, to treat flatulence, nausea and vomiting and has been used in tonics administered to convalescents (Grieve, 1994). Extracts from nutmeg have demonstrated a variety of biological activities including anti-inflammatory (Ozaki *et al.,* 1989), antimicrobial (Orabi *et al.,* 1991), metabolic

(Kumari and Rao, 1989; Singh and Rao, 1993b; Chhabra and Rao, 1994), anticancer (Hussain and Rao, 1991; Jannu *et al.,* 1991), and a variety of pharmaco-active (Kalbhen, 1971; Forrest and Heacock, 1972; Sherry *et al.,* 1982; Sharma *et al.,* 1995; Ram *et al.,* 1996) properties. A review of the toxicology and the pharmacology of nutmeg and its volatile oil has recently been published (McKee and Harden, 1991).

1.5.3.8. Oregano (*Origanum vulgare ssp. hirtum* (Link) J.H. Letsw.)

Synonyms: *Origanum heracleoticum* L.; *Origanum hirtum* Link.; *Origanum creticum* Sieber ex Bentham.

Family: Labiatae/Lamiaceae.

1.5.3.8.1. Botany

Origanum also called oregano or Mexican sage consists of the dried leaves and flowering tops of a variety of origanum vulgare L. a perennial (often grown as an annual) herbaceous plant belonging to the Labiatae/Lamiaceae, or mint family. It is indigenous to the sunny, sloping and hilly areas around the Mediterranean region and western Asia. The plant reaches a height of 1 to 2 feet and has ovate shaped, light brownish green leaves approximately 3 cm in length borne in clusters on short spikes.

1.5.3.8.2. Volatile Oil

The herb contains a volatile oil of aromatic, spicy, somewhat basil-like odour and fresh herbs yield 0.07% to 0.20% of oil, while the dried herb yields 0.15% to 0.40% oil. The major components have been reported to include α-pinene (0.7-2.7%), myrcene (0.5-2.9%), α-terpinene (0.6-1.8%), γ-terpinene (1.8-16.3%), *p*-cymene (4.9-24.9%), β-caryophyllene/terpinen-4-ol (0.6-2.3%), carvacrol (23.4-78.7%) and thymol (0.2-21.0%) and other minor components (Baser *et al.*, 1994).

1.5.3.8.3. Bioactivities.

The oil is reported to possess a number of ethnobotanical uses, including carminative, decongestant, diuretic, diaphoretic and emmengogue and sedative properties. The volatile oil has been reported as possessing a variety of biological activities including include antibacterial (Aureli *et al.,* 1992; Biondi *et al.,* 1993), antifungal (Akgül and Kivanc, 1988; Kivanc *et al.,* 1991; Stiles *et al.,* 1995),

insecticidal and antioxidant (Lagouri *et al.,* 1993; Dorman *et al.,* 1995a; Takácsová *et al.,* 1995; Aruoma *et al.,* 1996) properties.

1.5.3.9. Thyme (*Thymus vulgaris* L.)

Synonyms: Common Thyme; Garden Thyme.
Family: Labiatae/Lamiaceae.

1.5.3.9.1. Botany

Thymus vulgaris L. plants are hardy, diminutive perennial herbaceous shrubs native to the Mediterranean region and Asia Minor belonging to the Labiatae/Lamiaceae, or mint family. They can survive fairly harsh winter conditions but prefer a mild climate and a well-drained sunny location. They grow particularly well in light, chalky, stony soil. *Thymus vulgarus* of the garden type is sub-erect with numerous stems 20 to 45cm in height and has a woody fibrous root. The tiny greyish green narrow leaves rarely exceed 0.60cm in length and approximately 0.25cm in width.

1.5.3.9.2. Volatile Oil

The volatile oil of thyme is a pale, yellowish-red liquid with a rich sweet, aromatic, herbaceous odour yielding a sweet, phenolic odour. The yield is in the region of 2.0% to 2.5%. The representative composition (% composition range) of the volatile oil of thyme includes the monoterpenes *p*-cymene 2.2-42.8%, γ-terpinene 0.3-12.4%, α-pinene 0.9-3.7%, camphene 0.5-2.4%, myrcene trace-2.6%, α-terpinene 0.8-1.5%, limonene 0.4-2.1%, terpinolene trace-2%, α-thujene 0.5%, δ-3-carene 0.1%, sabinene 0.6%, α-phellandrene 0.1-0.2%, β-pinene (trace); the sesquiterpene β-caryophyllene 0.2-2.9%; the phenols thymol 30-48%, carvacrol 0.5-5% and methoxy-carvacrol (trace); the alcohols borneol trace-1.8%, linalool 1.3-12.4%, terpinen-4-ol 0.3-9.5%, α-terpineol 0.4-0.9%, geraniol 0.1-0.2%, β-terpineol 0.6-0.9% (*monoterpenols*) and nerolidol 0-0.8% (*sesquiterpenol*); the ketones camphor 2.3-16.3% and α-thujone 0.2%; the esters linalyl acetate 0.9%, α-terinyl acetate 0.7-1.4% and geranyl acetate 0-0.5% and oxides 1,8-cineole 0.4-7.4%, *trans*-linalool oxide 0.5% and *cis*-linalool oxide 1% (Williams, 1996).

1.5.3.9.3. Bioactivities

The medicinal action and uses of thyme volatile oil has been attributed it's variety of biological activities including antibacterial (Aureli *et al.,* 1992; Biondi *et al.,* 1993; Lattaoui and Tantaoui-Elaraki, 1994; Patákova and Chladek, 1974), antifungal (El-Maraghy, 1995; Llewellyn *et al.,* 1981), insecticidal (El-Gengaihi *et al.,* 1996; Farag *et al.,* 1994), antioxidant (Aruoma *et al.,* 1996; Dorman *et al.,* 1995a; Schwarz *et al.,* 1996) and pharmacological (Lemli, 1983) properties.

2. Plant Volatile Oil Composition Analysis Using Gas Chromatography and Mass Spectroscopy

2.1. Introduction

Volatile oils from aromatic and medicinal plants are mixtures of numerous phytochemicals in which compositional variations in terms of components and their relative amounts can occur in the oils extracted from plants of the same family and species and mode of extraction. This variation poses interesting challenges in determining the authenticity and degree of adulteration of bulk commercial supplies of volatile oils at the site of extraction, primary distribution and at retail. Gas chromatography - mass spectroscopy is a powerful technique in confirming a volatile oil's authenticity and its acceptability for its proposed use. However, its usefulness cannot be realized without an knowledge of a volatile oil's chemical composition and the variation range for each compound considered acceptable for such a test sample from its place of origin and relevant quality standards. In addition, it is important when screening plant volatile oils for bioactivities *in vitro* and understanding *in vivo* activity to know the chemical composition of the test material examined. This analysis is essential for determining which component or components possess an activity and to investigate the possibility of synergistic effects.

The aims of this present investigation were as to determine the chemical composition of the plant volatile oil samples.

2.2. Materials and Methods

2.2.1. Materials

Authentic plant volatile oil components were obtained from either Sigma Chemical Co. or Fluka Chemical Co. and heptane was obtained from Merck, Lutterworth, Liecester, UK.

2.2.2. Gas Chromatography - Mass Spectrometry

The GC-MS unit consisted of a Fissons MD-800D gas chromatograph equipped with a DB-1 fused-silica column (30m x 0.25mm id., film thickness 0.25μm; J & W Scientific Inc.) with an AS-800 autosampler, interfaced with a Fissons MD800 quadrupole analyzer. Oven temperature was programmed, 45°C - 175°C at a rate of 3°C min^{-1}, subsequently at 15°C min^{-1} up to 300°C, and held isothermal for 10 minutes. Injector, GC-MS interface and EI+ (electron impact ionisation) source temperatures were 250°C, 250°C and 200°C respectively. Helium was used as the carrier gas and was adjusted to a linear velocity of 30cm.s^{-1}. A 1μL aliquot was injected from the test sample (3μL oil, 1mL pentane) and underwent a 1:40 split ratio. Ionisation energy, 70eV; scan range 40 - 300 amu; scan time, 1s.

The identity of the components were assigned by comparison of their retention times with authentic samples (Appendix 1) and plant volatile oils of known composition and the mass spectra of an in-house customized database library and the NIST library (1995) using Masslab™ software (version 1.27, Fisons) and published data (Adams, 1995; Masada, 1975).

2.3. Results

2.3.1. The Chemical Composition of the Volatile Oil from Black Pepper

The identified components in the volatile oil of black pepper are listed in Table 10 and account for 83.89% of the oil. The total ionisation current chromatogram for black pepper can be seen in Appendix 3. The main components in this oil were the sesquiterpene hydrocarbons β-caryophyllene (**15**) (24.21%) and α-humulene (**31**) (2.16%), the oxygen-containing monoterpene hydrocarbons α-terpineol (**57**) (4.74%) and γ-terpineol (**59**) (1.48%). The remaining identified components included the monoterpene hydrocarbons limonene (**34**) (11.51%), δ-3-carene (**10**) (8.53%), β-pinene (**51**) (7.33%), α-pinene (**50**) (6.33%), α-phellandrene (**48**) (2.74%), α-copaene (**22**) (2.26%), *p*-cymene (**24**) (1.63%), the phenylpropanoid eugenol (**26**) (5.56%) and formic acid phenyl methyl ester (1.23%). A total of 201

peaks were detected of which 51 peaks were quantified as ≥0.1%. A total of 13 peaks were unidentified.

2.3.2. The Chemical Composition of the Volatile Oil from Clove

The identified components in the volatile oil of clove are listed in Table 11 and account for 98.47% of the oil. The total ionisation current chromatogram for clove volatile oil is shown in Appendix 4. The principal component of clove volatile oil was the phenylpropanoid eugenol (**26**) (86.48%). This was accompanied by the sesquiterpene hydrocarbon β-caryophyllene (**15**) (5.58%) and the ester eugenyl acetate (**27**) (5.15%). The remaining identified components included the sesquiterpene α-humulene (**31**) (0.66%), the oxygen-containing sesquiterpene caryophyllene oxide (**16**) (0.32%) and the hydrocarbon methyl salicylate (**40**) (0.28%). Two peaks were unidentified from the chromatogram (RT: 13.414; 0.1% and RT: 22.849; 0.11%). When the same volatile oil was run on a different GC-MS system, the two unidentified peaks and the component methyl salicylate (**40**) were not detected. A total of 98 peaks were detected of which 9 were quantified as ≥0.1%.

2.3.3. The Chemical Composition of the Volatile Oil from Geranium

The identified components in the volatile oil of geranium are shown in Table 12 and account for 75.58% of the oil. The total ionisation current chromatogram for geranium can be seen in Appendix 5. The main components were identified as the monoterpene aldehyde citronellal (**19**) (32.92%), the formate monoterpene ester citronellyl formate (**21**) (7.55%) and geranyl tiglate (**30**) (1.12%). The oxygen-containing monoterpenes were represented by *trans*-geraniol (**28**) (11.95%), linalool (**35**) (6.62%) and the ketones iso-menthone (**32**) (5.83%) and menthone (**38**) (1.75%). The sesquiterpene hydrocarbon β-caryophyllene (**15**) (1.20%) was also identified. A total of 167 peaks were detected of which 63 were quantified as ≥0.1%. A total of 30 peaks were unidentified.

Table 10. The percentage composition of the plant volatile oil from black pepper.

	Black Pepper	
Component	**RT [min]**	**%Area**
α-Thujene	8.862	0.44
α-Pinene	9.102	6.33
Camphene	9.582	0.38
Sabinene	10.543	0.15
β-Pinene	10.838	7.33
α-Phellandrene	11.853	2.74
δ-3-Carene	12.213	8.53
α-Terpinene	12.414	0.35
p-Cymene	12.534	1.63
Limonene	13.146	11.51
β-Terpineol	18.726	0.34
Terpinen-4-ol	19.517	0.24
α-Terpineol	20.047	4.74
γ-Terpineol	20.397	1.48
Neral	22.158	0.32
Geraniol	23.509	0.54
t-Anethole	24.259	0.39
Eugenol	27.401	5.56
α-Cubebene	28.311	0.23
α-Copaene	29.452	2.26
β-Elemene	30.052	0.37
β-Caryophyllene	31.233	24.21
Aromadendrene	32.243	0.47
α-Humulene	32.623	2.16
δ-Cadinene	35.465	0.35
Caryophyllene oxide	37.536	0.84
% Identification:	-	83.89%
Monoterpene Hydrocarbons:	-	39.39%
Oxygen-containing Monoterpenes:	-	7.66%
Sesquiterpene Hydrocarbons:	-	30.05%
Oxygen-containing Sesquiterpenes:	-	0.84%
Phenylpropanoids:	-	5.95%

RT: Retention time. 201 peaks in total; 51 peaks > 0.1%; 13 peaks not identified. 11 peaks (Pinane: NIST 7100; Formic acid phenyl methyl ester: NIST 6476; β-Terpineol: NIST 10925; γ-Terpineol: NIST 10908; α-Cubebene: NIST 23919; Caryophyllene Oxide: NIST 27701) identified from the NIST library (1994).

Table 11. The percentage composition of the plant volatile oil from clove.

	Clove	
Component	**RT [min]**	**%Area**
Methyl salicylate	19.828	0.28
Eugenol	27.491	86.48
β-Caryophyllene	31.223	5.58
α-Humulene	32.624	0.66
Eugenyl acetate	34.385	5.15
Caryophyllene oxide	37.537	0.32
% Identification	-	98.47%
Monoterpene Hydrocarbons	-	-
Oxygen-containing Monoterpenes	-	-
Sesquiterpene Hydrocarbons	-	6.24%
Oxygen-containing Sesquiterpenes	-	0.32%
Phenylpropanoids	-	86.48%
Others	-	0.28%

RT: Retention time. 98 peaks in total; 9 peaks > 0.1%; 2 peaks not identified. 2 peaks (Methyl Salicylate: NIST 10159 and Caryophyllene Oxide: NIST 27701) identified from the NIST library (1994).

2.3.4. The Chemical Composition of the Volatile Oil from Lovage Leaf (3 months)

The identified components in the volatile oil of lovage leaf (3 months) are listed in Table 13 and account for 65.46% of the oil. The total ionisation current chromatogram for lovage leaf can be seen in Appendix 6. The composition of the volatile oil extracted from lovage leaf on GC-MS analysis revealed the following main components (≥0.1%): *p*-menth-1-en-8-ol acetate (**39**) (58.35%), unknowns: [RT: 12.952] (17.65%), [RT: 42.510] (12.29%), myrcene (**41**) (2.32%), α-terpineol (**57**) (1.49%), unknown [RT:44.477] (1.03%), α-pinene (**50**) (0.75%), α-phellandrene (**48**) (0.64%), neryl acetate (**46**) (0.61%), terpinolene (**60**)(0.38%), unknowns: [RT: 19.070] (0.26%), [RT: 33.824] (0.25%), sabinene (**52**) (0.22%), camphene (**11**)(0.21%), unknown [RT:40.226] (0.17%), β-pinene (**51**) (0.19%), unknown [RT:41.843] (0.17%), terpinen-4-ol (**56**) (0.16%), unknown [RT: 24.738] (0.16%), γ-terpinene (**55**) (0.14%) and unknowns: [RT: 23.288; 25.305] (0.14%), [RT: 11.152] (0.13%), [RT: 18.853] (0.12%) and [RT: 19.920] (0.10%).

Table 12. The percentage composition of the plant volatile oil from geranium.

	Geranium	
Component	**RT [min]**	**%Area**
α-Pinene	9.132	0.54
Myrcene	11.383	0.17
p-Cymene	12.553	0.15
Limonene	12.994	0.21
trans-β-Ocimene	13.874	0.13
cis-Linalool oxide	14.695	0.29
trans-Linalool oxide	15.375	0.16
Linalool	16.015	6.62
Menthone	18.137	1.75
iso-Menthone	18.557	5.83
α-Terpineol	20.058	0.57
Citronellal	22.109	32.92
Trans-Geraniol	23.219	11.95
Geraniol	23.52	0.60
Citronellyl formate	24.32	7.55
Citronellyl acetate	27.912	0.33
α-Cubebene	28.312	0.20
Geranyl acetate	29.072	0.44
α-Copaene	29.463	0.51
β-Caryophyllene	31.224	1.20
Aromadendrene	32.224	0.41
α-Humulene	32.634	0.25
Allo-Aromadendrene	32.954	0.16
β-Selinene	33.965	0.12
Bicyclogermacrene	34.425	0.65
α-Muurolene	34.595	0.21
δ-Cadinene	35.095	0.20
Calamenene	35.246	0.34
Geranyl Tiglate	42.089	1.12
% Identification:	-	75.55%
Monoterpene Hydrocarbons:	-	1.20%
Oxygen-containing Monoterpenes:	-	70.10%
Sesquiterpene Hydrocarbons:	-	4.25%
Oxygen-containing Sesquiterpenes:	-	-
Others:	-	-

RT: Retention time. 167 peaks in total; 68 peaks > 0.1%; 30 peaks not identified. 6 peaks (Citronellyl Acetate: NIST 18879; Cadinene: NIST 23957 and Geranyl Tiglate: NIST 31133) identified from the NIST library (1994).

2.3.5. The Chemical Composition of the Volatile Oil from Lovage Leaf (6 Months)

The identified components in the volatile oil of lovage leaf (6 months) are listed in Table 14 and account for 61.05% of the oil. The total ionisation current chromatogram for lovage leaf can be seen in Appendix 7. The main components (≥0.1%) in this oil were *p*-menth-1-en-8-ol acetate (**39**) (52.78%), unknowns: [RT: 12.935] (22.51%), [RT: 42.492] (12.58%), myrcene (**41**) (2.96%), α-terpineol (**57**) (1.27%), α-pinene (**50**) (0.85%), α-phellandrene (**48**) (0.83%), neryl acetate (**46**) (0.62%), unknown [RT: 44.476] (0.39%), terpinen-4-ol (**56**) (0.37%), terpinolene (**60**) (0.36%), unknowns: [RT:33.823] (0.28%), [RT:19.070] (0.25%), β-pinene (**51**) (0.24%), γ-terpinene (**55**) (0.24%), camphene (**11**) (0.23%), sabinene (**52**) (0.22%), unknowns: [RT: 40.225] (0.21%), [RT: 18.853] (0.19%), [RT: 19.920; 25.288] (0.18%), [RT: 41.842] (0.15%), [RT: 24.738; 40.475] (0.12%), [RT: 23.517] (0.115) and *p*-cymene (**24**) (0.10%).

Table 13. The percentage composition of the plant volatile oil from lovage leaf (3 months).

	Lovage (Leaf)	
Component	**RT [min]**	**%Area**
α-Pinene	9.168	0.75
Camphene	9.651	0.21
Sabinene	10.601	0.22
β-Pinene	10.752	0.19
Myrcene	11.418	2.32
α-Phellandrene	11.918	0.64
γ-Terpinene	14.352	0.14
Terpinolene	15.736	0.38
Terpinen-4-ol	19.587	0.16
α-Terpineol	20.104	1.49
p-Menth-1-en-8-ol acetate	27.773	58.35
Neryl acetate	29.140	0.61
% Identification:		65.46%
Monoterpene Hydrocarbons:		4.85%
Oxygen-containing Monoterpenes:		60.61%
Sesquiterpene Hydrocarbons:		-
Oxygen-containing Sesquiterpenes:		-
Others:		-

RT: Retention Time.

Table 14. The percentage composition of the plant volatile oil from lovage leaf (6 months).

	Lovage Leaf	
Component	**RT [min]**	**%Area**
α-Pinene	9.168	0.85
Camphene	9.635	0.23
Sabinene	10.601	0.22
β-Pinene	10.735	0.24
Myrcene	11.418	2.96
α-Phellandrene	11.918	0.83
p-Cymene	12.602	0.10
γ-Terpinene	14.336	0.24
Terpinolene	15.719	0.36
Terpinen-4-ol	19.570	0.37
α-Terpineol	20.104	1.27
p-Menth-1-en-8-ol acetate	27.739	52.78
Neryl acetate	29.139	0.62
% Identification:	-	61.05%
Monoterpene Hydrocarbons:	-	6.01%
Oxygen-containing Monoterpenes:	-	55.04%
Sesquiterpene Hydrocarbons:	-	-
Oxygen-containing Sesquiterpenes:	-	-
Others:	-	-

RT: Retention time.

2.3.6. The Chemical Composition of the Volatile Oil from Lovage Stem

The identified components in the volatile oil of lovage stem are listed in Table 15 and account for 69.18% of the oil. The total ionisation current chromatogram for lovage stem can be seen in Appendix 8. The main components (≥0.1%) in this oil were α-terpinyl acetate (**61**) (56.18%), unknowns: [RT: 12.952] (14.74%), [RT: 42.493] (7.86%), α-pinene (**50**) (2.79%), myrcene (**41**) (2.04%), β-pinene (**51**) (1.76%), unknown [RT: 40.209] (1.70%), α-terpineol (**57**) (1.21%), camphene (**11**) (1.06%), neryl acetate (**46**) (1.00%). Terpinen-4-ol (**56**) (0.81%), bornyl acetate (**7**) (0.67%), *p*-cymene (**24**) (0.61%), γ-terpinene (**55**) (0.59%), unknown [RT: 34.774] (0.42%), terpinolene (**60**) (0.37%), unknowns: [RT: 25.288; 41.842] (0.33%), [RT: 19.187; 35.140] (0.32%), α-phellandrene (**48**) (0.28%), unknown [RT: 40.509]

(0.25%), α-thujene (**62**) (0.21%), α-terpinene (**54**) (0.19%), unknowns: [RT: 21.821; 44.476] (0.18%), [RT: 15.852; 38.708] (0.15%), [RT: 14.469] (0.14%), [RT: 23.588; 36.124] (0.13%), [RT: 16.419] (0.12%), [RT: 28.939; 30.806] (0.11%), [RT: 18.803; 23.788; 25.622; 30.506] (0.10%).

Table 15. The percentage composition of the plant volatile oil from lovage stem.

	Lovage Stem	
Component	**RT [min]**	**%Area**
α-Thujene	8.918	0.21
α–Pinene	9.168	2.79
Camphene	9.651	1.06
β–Pinene	10.751	1.76
Myrcene	11.418	2.04
α-Phellandrene	11.918	0.28
α-Terpinene	12.485	0.19
p-Cymene	12.602	0.61
γ-Terpinene	14.352	0.59
Terpinolene	15.736	0.37
Terpinen-4-ol	19.587	0.81
α-Terpineol	20.104	1.21
Bornyl acetate	24.755	0.67
α-Terpinyl acetate	27.755	56.18
Neryl acetate	29.139	1.00
% Identification:	-	69.18%
Monoterpene Hydrocarbons:	-	9.31%
Oxygen-containing Monoterpenes:	-	59.87%
Sesquiterpene Hydrocarbons:	-	-
Oxygen-containing Sesquiterpenes:	-	-
Others:	-	-

RT: Retention time.

2.3.7. The Chemical Composition of the Volatile Oil from Melissa

The identified components in the volatile oil of melissa are shown in Table 16 and account for 96.54% of the oil. The total ionisation current chromatogram for melissa can be seen in Appendix 9. This oil was identified as being mainly composed of monoterpene hydrocarbons (59.02%) principally limonene (**34**) (57.53%) and oxygen-containing monoterpenes hydrocarbons (37.52%) mainly citronellal (**19**)

(24.92%). A number of unidentified compounds were detected at appreciable levels. These included an [RT: 26.256] (0.25%), [RT: 17.453] (0.20%), [RT: 20.904] (0.14%), [RT: 20.121] (0.13%), [RT: 11.318; 12.269; 19.121] (0.12%) and [RT: 19.537] (0.10%).

Table 16. The percentage composition of the plant volatile oil from melissa.

	Melissa	
Component	**RT [min]**	**%Area**
α-Pinene	9.168	0.35
Sabinene	10.601	0.52
Myrcene	11.418	0.62
Limonene	13.069	57.53
Linalool	16.070	0.60
Isopulegol	18.045	1.93
Citronellal	18.320	24.92
Nerol	22.221	4.34
Geraniol	23.572	5.73
% Identification:	-	96.54%
Monoterpene Hydrocarbons:	-	59.02%
Oxygen-containing Monoterpenes:	-	37.52%
Sesquiterpene Hydrocarbons:	-	-
Oxygen-containing Sesquiterpenes:	-	-
Phenols:	-	-
Others:	-	-

RT: Retention time.

2.3.8. The Chemical Composition of the Volatile Oil from Monarda

The identified components in the volatile oil of monarda are shown in Table 17 and account for 95.86% of this oil. The total ionisation current chromatogram for monarda can be seen in Appendix 10. This oil could be divided into five fractions: monoterpene hydrocarbons (14.18%), oxygenated monoterpene hydrocarbons (2.20%), sesquiterpenes (0.89%), phenolic hydrocarbons (76.71%) and others (1.88%). The principal fraction consisted of the phenolic hydrocarbons thymol (**48**) (70.59%) and carvacrol (**10**) (6.12%). The monoterpene hydrocarbons were mainly represented by *p*-cymene (**24**) (10.63%), with the remaining members being minor (<1.0%) components, while terpinen-4-ol (**42**) was the most abundant oxygenated monoterpene hydrocarbon (1.23%). The sesquiterpenes fraction was identified as β-

caryophyllene (**15**) (0.64%) and δ-cadinene (**8**) (0.25%). The hydrocarbon 7-octen-4-ol was identified at the appreciable level of 1.19%. A number of unknown components were not identified: [RT: 37.642] (0.25%), [RT: 14.470] (0.21%), [RT: 33.691] (0.20%), [RT: 28.323] (0.18%)[RT: 14.703; 35.175] (0.15%), [RT: 15.837] (0.14%).

Table 17. The percentage composition of the plant volatile oil from monarda.

	Monarda	
Component	**RT [min]**	**%Area**
α-Thujene	8.901	0.16
α-Pinene	9.151	0.28
7-Octen-4-ol	10.585	1.19
Myrcene	11.402	0.91
α-Terpinene	12.469	0.88
p-Cymene	12.636	10.63
1,8-Cineole	12.919	0.26
Limonene	13.019	0.38
γ-Terpinene	14.336	0.94
Linalool	16.053	0.30
Borneol	18.937	0.27
Terpinen-4-ol	19.588	1.23
α-Terpineol	20.238	0.14
Carvacrol methyl ether	22.789	0.69
Thymol	25.206	70.59
Carvacrol	25.439	6.12
β-Caryophyllene	31.307	0.64
δ-Cadinene	35.592	0.25
% Identification:	-	95.86%
Monoterpene Hydrocarbons:	-	14.18%
Oxygen-containing Monoterpenes:	-	2.20%
Sesquiterpene Hydrocarbons:	-	0.89%
Oxygen-containing Sesquiterpenes:	-	-
Phenols:	-	77.40%
Others:	-	1.19%

RT: Retention time.

2.3.9. The Chemical Composition of the Volatile Oil from Nutmeg

The identified components in the volatile oil of nutmeg are listed in Table 18 and account for 92.01% of the oil. The total ionisation current chromatogram for nutmeg can be seen in Appendix 11. The main components were the monoterpene hydrocarbons α-pinene (**50**) (22.04%), β-pinene (**51**) (21.53%), sabinene (**29**) (15.39%), limonene (**34**) (3.87%), myrcene (**29**) (1.89%), *p*-cymene (**24**) (1.87%), γ-terpinene (**41**) (1.80%), α-terpinene (**40**) (1.24%), α-thujene (**47**) (1.18%) and the oxygen-containing monoterpene hydrocarbon terpinen-4-ol (**42**) (5.72%) and myristicin (**30**) (9.43%). A total of 135 peaks were detected of which 34 were quantified as ≥0.1%.

2.3.10. The Chemical Composition of the Volatile Oil from Nutmeg [Serva]

The components identified in the volatile oil of nutmeg [*Serva*] are listed in Table 19 and account for 89.29% of the oil. The total ionisation current chromatogram for nutmeg can be seen in Appendix 12. This oil principally consisted of monoterpene hydrocarbons (76.87%) of which 15.15% were oxygenated monoterpenes. The remaining components included sesquiterpene hydrocarbons (5.11%) and the phenylpropanoid eugenol (**26**) (3.22%). The main monoterpenes were determined as sabinene (**39**) (13.59%), α- and β-pinene (**50**, **51**) (11.39% and 9.39% respectively), limonene (**34**) (10.00%) and γ-terpinene (**41**) (2.19%). Chiefs amongst the oxygen-containing monoterpene hydrocarbons were the alcohols α-terpineol (**57**) (6.78%), terpinen-4-ol (**42**) (4.85%), γ-terpineol (**59**) (1.63%) and linalool (**35**) (1.20%). Geraniol (**28**) and borneol (**5**) were minor components at 0.70% and 0.34% respectively. Sesquiterpene hydrocarbons included appreciable amounts of β-caryophyllene (**15**) (4.83%) and its oxide, caryophyllene oxide (**16**) (0.28%) with α-humulene (**31**) content <1.0%. Myristicin (**30**) was identified at 2.64%.

Table 18. The percentage composition of the plant volatile oil from nutmeg.

	Nutmeg	
Component	**RT [min]**	**%Area**
α-Thujene	8.882	1.18
α-Pinene	9.132	22.04
Camphene	9.602	0.37
Sabinene	10.573	15.39
β-Pinene	10.713	21.53
Myrcene	11.373	1.89
α-Phellandrene	11.873	0.67
δ-3-Carene	12.224	0.89
α-Terpinene	12.434	1.24
p-Cymene	12.554	1.87
Limonene	12.984	3.87
γ-Terpinene	14.295	1.80
Terpinolene	15.676	0.93
Linalool	16.016	0.47
Borneol	18.877	0.25
Terpinen-4-ol	19.528	5.72
α-Terpineol	20.058	0.73
trans-Geraniol	23.24	0.13
Bornyl acetate	24.69	0.11
Eugenol	27.412	0.35
α-Terpinyl acetate	27.652	0.14
Neryl acetate	29.083	0.15
Methyl eugenol	29.443	0.58
Myristicin	34.375	9.43
Myristic acid methyl ester	43.41	0.28
% Identification:	-	92.01%
Monoterpene Hydrocarbons:	-	73.67%
Oxygen-containing Monoterpenes:	-	7.70%
Sesquiterpene Hydrocarbons:	-	-
Oxygen-containing Sesquiterpenes:	-	-
Phenylpropanoids:	-	0.93%
Others:	-	9.71%

RT: Retention time. 135 peaks in total; 34 peaks > 0.1%; 5 peaks not identified. 4 peaks (Myristic acid methyl ester: NIST 32399) identified from the NIST library (1994).

Table 19. The percentage composition of the plant volatile oil from nutmeg [Serva].

	Nutmeg [Serva]	
Component	**RT [min]**	**%Area**
Tricyclene	8.692	0.75
α-Thujene	8.882	1.00
α-Pinene	9.132	11.39
Camphene	9.602	3.76
Sabinene	10.573	13.59
β-Pinene	10.703	9.39
Myrcene	11.373	1.24
α-Phellandrene	11.883	0.51
α-Terpinene	12.434	1.71
p-Cymene	12.554	3.94
Limonene	12.994	10.00
γ-Terpinene	14.305	2.19
Terpinolene	15.685	1.90
Linalool	16.026	1.20
cis-β-Terpineol	18.737	0.20
Borneol	18.877	0.34
Terpinen-4-ol	19.528	4.85
α-Terpineol	20.058	6.78
γ-Terpineol	20.418	1.63
Linalool formate	21.519	0.15
trans-Geraniol	23.220	0.70
Eugenol	27.412	3.22
α-Cubebene	28.313	0.17
β-Caryophyllene	31.224	4.83
Aromadendrene	32.495	0.14
α-Humulene	32.635	0.61
Myristicin	34.376	2.64
δ-Cadinene	35.506	0.18
Caryophyllene oxide	37.557	0.28
% Identification:	-	89.29%
Monoterpene Hydrocarbons:	-	61.37%
Oxygen-containing Monoterpenes:	-	15.85%
Sesquiterpene Hydrocarbons:	-	5.93%
Oxygen-containing Sesquiterpenes:	-	0.28%
Phenylpropanoids:	-	3.22%
Others:	-	2.64%

2.3.11. The Chemical Composition of the Volatile Oil from Oregano

The identified components in the volatile oil of oregano are listed in Table 20. These accounted for 93.30% of the volatile oil. The total ionisation current chromatogram for oregano can be seen in Appendix 13. Four fractions were identified: phenolic hydrocarbons (70.73%), monoterpene hydrocarbons (18.69%), oxygenated monoterpenes (3.32%) and a hydrocarbon fraction (0.29%) composed of the alkene styrene and the alcohol 7-octen-4-ol. The principal component in this volatile oil was the phenolic hydrocarbon carvacrol (**10**) (69.37%). Thymol (**48**) was identified at a significantly lower amount (1.36%). The monoterpene hydrocarbon *p*-cymene (**24**) was identified as the second most abundant component at 12.70% follow by γ-terpinene (**41**) (2.09%), myrcene (**29**) (1.45%) and α-terpinene (**40**) (1.01%). The remaining monoterpenes and the oxygen-containing monoterpene hydrocarbons were identified as minor (<1.0%) constituents of this oil. The composition was found to be in general agreement with the literature (Prakash 1991) A total of 176 peaks were detected of which 36 were quantified as ≥0.1%.

2.3.12. The Chemical Composition of the Volatile Oil from Thyme

The percentage composition of identified components in the volatile oil of thyme are listed in Table 21 and accounting for 98.86% of the oil. The total ionisation current chromatogram for thyme can be seen in Appendix 14. The principal fraction was phenolic (50.95%) followed by monoterpene hydrocarbons (36.76%), oxygen-containing monoterpene hydrocarbons (9.17%) and the sesquiterpene β-caryophyllene (**16**) (0.91%). The main phenolic hydrocarbon was identified as thymol (**48**) (48.00%), with appreciable levels of carvacrol (**10**) (2.95%). The most abundant monoterpene was *p*-cymene (**24**) (25.51%) followed by γ-terpinene (**41**) (4.44%), α-pinene (**50**) (2.12%), myrcene (**29**) (1.22%) and limonene (**34**) (1.14%). The remaining monoterpene hydrocarbons were identified as minor (<1.0%) components. The principal oxygen-containing monoterpenes were identified as linalool (**35**) (4.56%), the ester bornyl acetate (**6**) (1.91%) and the oxide 1,8-cineole (**14**) (1.25%). The alcohols α- and γ-terpineol (**57**, **59**) and terpinen-4-ol (**42**)

were an identified as minor (<1.0%) components. A total of 106 peaks were detected of which 28 peaks were identified as ≥0.1%.

Table 20. The percentage composition of the plant volatile oil from oregano.

	Oregano	
Component	**RT [min]**	**%Area**
Camphene	9.603	0.53
7-octen-4-ol	10.573	0.17
Myrcene	11.374	1.45
δ-3-carene	12.234	0.14
α-Terpinene	12.434	1.01
p-cymene	12.554	12.70
1,8-Cineole	12.874	0.45
Limonene	12.984	0.77
γ-Terpinene	14.295	2.09
Styrene	15.396	0.12
Terpinolene	15.686	0.27
Linalool	16.016	0.61
Borneol	18.878	0.43
Terpinen-4-ol	19.528	0.82
α-Terpineol	20.058	0.75
γ-Terpineol	20.428	0.15
Bornyl acetate	24.711	0.11
Thymol	24.871	1.36
Carvacrol	25.271	69.37
% Identification:	-	93.30%
Monoterpene Hydrocarbons:	-	18.96%
Oxygen-containing Monoterpenes:	-	3.32%
Sesquiterpene Hydrocarbons:	-	-
Oxygen-containing Sesquiterpenes:	-	-
Phenols:	-	70.73%
Others:	-	0.29%

RT: Retention time. 176 peaks in total; 36 Peaks > 0.1%; 14 peaks not identified. 3 peak (camphor: NIST 10945) identified from the NIST library (1994).

Table 21. The percentage composition of the plant volatile oil from thyme.

	Thyme	
Component	**RT [min]**	**%Area**
α-Thujene	8.892	0.14
α-Pinene	9.132	2.12
Camphene	9.602	0.17
β-Pinene	10.703	0.10
Myrcene	11.383	1.22
α-Terpinene	12.434	0.44
p-Cymene	12.564	25.51
1,8-Cineole	12.884	1.25
Limonene	12.994	1.14
γ-Terpinene	14.305	4.44
Terpinolene	15.686	0.49
Linalool	16.026	4.56
Camphor	17.447	0.13
Borneol	18.877	0.86
Terpinen-4-ol	19.538	0.23
α-Terpineol	20.058	0.94
β-Terpineol	20.428	0.28
Bornyl acetate	24.710	1.91
Thymol	24.881	48.00
Carvacrol	25.221	2.95
β-Caryophyllene	31.234	0.98
% Identification:	-	97.86%
Monoterpene Hydrocarbons:	-	36.76%
Oxygen-containing Monoterpenes:	-	9.17%
Sesquiterpene Hydrocarbons:	-	0.98%
Oxygen-containing Sesquiterpenes:	-	-
Phenols:	-	50.95%
Others:	-	-

RT: Retention time. 106 peaks in total; 28 Peaks > 0.1%; 5 peaks not identified. 1 peak (camphor: NIST 10945) identified from the NIST library (1994).

2.4. Conclusions

From the data presented in this chapter, a number of points become apparent.

1. Plant oils used in this study are mixtures of often a large number of components.
2. Oils from different plant species may share similar components, e.g. α-pinene (Tables 10, 12– 17, 19 and 21), β-caryophyllene (Tables 10-12, 17,19 and 21) and α-humulene (Tables 10-12 and 19).
3. The components are representative of different chemical classes:

- Aliphatic (acyclic) terpenes: myrcene (Tables 10, 12-21).
- Monocyclic terpenes: terpinenes (Tables 10, 15, 17-21 [α-]/Tables 13-15, 17-21 [γ-]) and α-phellandrene (Tables 10, 13-15, 18-19).
- Bicyclic terpenes: pinenes (Tables 10, 12-21 [α-], Tables 13-15, 18, 19, 21 [β-]); δ-3-carene (Tables 10, 18 and 20) and camphene (Tables 10, 13-15, 18-21).
- Sesquiterpenes: β-caryophyllenes (Tables 10-12, 17, 19 and 21).
- Terpenoid aldehydes: neral (Table 10), citronellal (Tables 12 and 16).
- Terpenoid ketones: menthone (Table 12); *iso*-menthone (Table 12).
- Aliphatic alcohols: 7-octen-4-ol (Tables 17 and 20).
- Acyclic alcohols: linalool (Tables 12, 16-21) and geraniol (Tables 10, 12 and 16).
- Cyclic alcohols: terpineols (Tables 10, 12-15, 17-21 [α-]; Table 10 [β-] and Tables 10, 19-21 [γ-]) and terpinen-4-ol (Tables 10, 13-15, 17-21).
- Phenols: carvacrol (Tables 17, 20 and 21) and thymol (Tables 17, 20 and 21); the phenylpropanoids: *t*-anethole (Table 10); eugenol (Tables 10, 11, 18 and 19).
- Esters:

  (a) Aliphatic (acyclic): geranyl acetate (Table 12); citronellyl acetate (Table 12); neryl acetate (Tables 13-15 and 18).

  (b) Cyclic esters: *p*-menth-1-en-8-ol acetate [terpinyl acetate] (Tables 13-16 and 18); myristic acid methyl ester (Table 18).

  (c) Bicyclic ester: bornyl acetate (Tables 15, 18, 20 and 21).

- Ethers: carvacrol methyl ether (Table 17).
- Miscellaneous/others: 1,8-cineole (Tables 17, 20 and 21); linalool oxide (Table 12); caryophyllene oxide (Tables 10, 11 and 19).

3. Antibacterial Effects of Selected Plant Volatile Oils and Their Phytoconstituents upon a Range of Bacterial Organisms

3.1. Introduction

The antiseptic qualities of aromatic and medicinal plants and their extracts have been anecdotally recognized since antiquity while attempts to characterise these properties in the laboratory date back to the early 1900's (Martindale, 1910; Hoffman and Evans, 1911; Rippetoe and Wise, 1912; Bachmann, 1916; Bachmann, 1918). The antimicrobial properties of plant volatile oils and constituents from a wide variety of plants have been assessed (Carson *et al.,* 1995; Carson *et al.,* 1996; Crespo *et al.,* 1990; Cruz *et al.,* 1989; Deans and Svoboda, 1988; Deans and Svoboda, 1989; Garg and Dengre 1986; Jain and Kar, 1971; Larrondo *et al.,* 1995; Lis-Balchin *et al.,* 1998; Low *et al.,* 1974; Nenoff *et al.,* 1996; Onawunmi *et al.,* 1984; Pattnaik *et al.,* 1995; Recio *et al.,* 1989; Ríos *et al.,* 1987; Sherif *et al.,* 1987; Vanhaelen-Fastré, 1973) and reviewed (Janssen *et al.,* 1987; Ríos *et al.,* 1988).

It is clear from these studies that these plant secondary metabolites have potential in medical procedures and applications in the cosmetic, food (Baratta *et al.,* 1998a; Baratta *et al.,* 1998b; Baratta *et al.,* 1998c; Gallardo *et al.,* 1987; Jay and Rivers, 1984; Shelef, 1983; Ueda *et al.,* 1982; Youdim *et al.,* 1998) and pharmaceutical (Cai and Wu, 1996; Janssen *et al.,* 1988, Pélissier *et al.,* 1994; Shapiro *et al.,* 1994) industries.

Investigations into the antimicrobial activities, mode of action and potential uses of plant volatile oils has re-gained momentum. There is appears to be a revival in the use of traditional approaches to protecting livestock and food from disease, pests and spoilage in industrial countries. This is especially true in regard to plant volatile oils and their antimicrobial evaluation, as can be seen from the comprehensive range of organisms volatile oils have been tested against. These have included: food spoiling organisms (Connor and Beuchat, 1984a; Connor and Beuchat, 1984b; Janssen *et al.,* 1988; Ouattara *et al.,* 1997; Zaika *et al.,* 1983) and

food poisoning organisms (Beuchat, 1976; Deans and Ritchie, 1987; Lis-Balchin and Deans, 1997; Tharib *et al.,* 1983) spoilage and mycotoxigenic filamentous fungi (Knobloch *et al.,* 1989), pathogenic and dimorphic yeasts (Boonchild and Flegel, 1982; Ghannoum, 1988) and animal and plant viruses (Ieven *et al.,* 1982; Romerio *et al.,* 1989; van den Berghe *et al.,* 1978).

The volatile oils of black pepper, clove, geranium, melissa, nutmeg, oregano and thyme were screened for antimicrobial activity using an agar diffusion technique (Deans and Ritchie, 1987) against 25 microorganisms of significant importance, Table 22. In addition, thirty-three authentic terpenoids and the phenylpropanoid eugenol commonly found in these volatile oils (Chapter 2), were also screened for activity. A selection of antibiotics (chloramphenicol (10μg), erythromycin (10μg), sulphafuragole (100μg), novobiocin (5μg), oleandomycin (5μg), penicillin (1.5μg), streptomycin (10μg), tetracycline (10μg), neomycin (10μg) and ampicillin (10μg)) were also assessed for comparison purposes using a disk method (Ríos *et al.,* 1988), Table 23.

The aims of this present investigation were as follows:

1. To assess the antimicrobial activities of the test volatile oils and compare them to the effect of the antibiotics upon bacterial growth.

2. To assess the components determined to be present in the volatile oils (Chapter 2) where available.

3. To use these data to deduce which components are likely to explain the activities of the oils.

4. Determine where possible any structural relationships between the components and their antibacterial activity.

3.2. Materials and Methods

3.2.1. Materials

Anaerobic indicator strips, Iso-Sensitest agar [CM 471] and gas generating kits were purchased from Oxoid, Unipath Ltd., Hampshire, UK. Vernier calipers

were purchased from Mauser, Germany while the Pharmacia gel punch was purchased from Uppsala, Sweden.

3.2.2. Bacterial Strains

Twenty five bacterial strains were used to assess the antibacterial properties of the test samples, 9 Gram positive and 16 Gram negative bacteria. Twenty-four out of twenty five bacterial strains were maintained on Iso-Sensitest agar slopes [CM 471] at room temperature. *Clostridium sporogenes* was maintained on cooked meat broth under anaerobic conditions. All strains were subcultured every two weeks. The sources of the strains used are listed in Table 22.

3.2.3. Assessment of Inhibition of Bacterial Growth

The measurement of growth inhibition was carried out in agreement with the method of Deans and Ritchie (1987). Iso-Sensitest agar was made up by dissolving 31.4g of agar in 1L of distilled water. 10mL amounts of molten agar were added to universal bottles and allowed to set at an angle to produce a slope. Agar for the test plates was stored in medical flats in 100mL amounts, this being sufficient for five plates. Iso-Sensitest broth was made up by dissolving 23.4g of powder in 1 litre of distilled water. 10mL amounts of the broth were autoclaved at 121°C for 15 minutes and used within one week of reconstitution.

Cells from cultures grown on Iso-Sensitest slopes were inoculated using a sterile loop into fresh Iso-Sensitest broth and incubated overnight at 25°C [10mL volume, $10^6 mL^{-1}$ final concentration]. In the case of the *Clostridium* culture, a universal containing a 20mL solution of meat extract broth was boiled for 20 minutes and allowed to cool in order to create anaerobic conditions, then was incubated with a loopful of broth from the original inoculated culture. 1mL amounts of each culture were then pipetted into separate sterile Petri dishes to which 20mL amounts of molten Iso-Sensitest agar [45°C] were added. The plates were then agitated to ensure mixing of the agar with the bacterial suspension. Once set, wells of 4mm diameter were then made in the center of each agar plate using a Pharmacia gel punch

[Uppsala, Sweden]. The plates were then left undisturbed to allow diffusion of the sample throughout the agar, and then incubated at 25°C for 48 hours.

The *Clostridium* plates were placed in an anaerobic jar containing a catalyst and a gas generating kit. Each sachet contained tablets of sodium borohydride, tartaric acid and sodium bicarbonate. When 10mL of distilled water was added to the sachet, approximately 100mL of hydrogen and 350mL of carbon dioxide was produced. An anaerobic indicator strip was also placed in the jar prior to incubation. Inhibition zones were measured using Vernier calipers. The evaluation of antibacterial activity was carried out using aseptic technique at all times and in duplicate with two replicates on every occasion.

3.2.4. Statistical Analysis

Statistical analysis included the calculation of the mean of the measured values and the standard error of the mean (S.E.M.) in agreement with published data. All statistical analyses were carried out using Minitab® for Windows 32Bit Release 10.5 Xtra software.

Table 22. Test bacteria and their source.

Organism	Source[a]	Gram Type	Classification
Acinetobacter calcoacetica	NCIB 8250	negative	Food Spoilage
Aeromones hydrophila	NCTC 8049	negative	Food Spoilage
Alcaligenes faecalis	NCIB 8156	negative	Food Spoilage
Bacillus subtilis	NCIB 3610	positive	Food Spoilage
Beneckea natriegens	ATCC 14048	negative	Environmental
Brevibacterium linens	NCIB 8456	positive	2° Pathogen/Opportunistic
Brocothrix thermosphacta	Sausage meat	positive	Food Spoilage
Citrobacter freundii	NCIB11490	negative	2° Pathogen
Clostridium sporogenes	NCIB 10696	positive	Food Poisoning
Enterococcus faecalis	NCTC 775	positive	Human Pathogen
Enterobacter aerogenes	NCTC10006	negative	Coliform
Erwinia carotovora	NCPPB 312	negative	Plant Pathogen
Escherichia coli	NCIB 8879	negative	Commensal/Human Pathogen
Flavobacterium suaveolens	NCIB 8992	negative	Food Spoilage
Klebsiella pneumoniae	NCIB 418	negative	Human Pathogen
Lactobacillus plantarum	NCDO 343	positive	Food Spoilage
Leuconostoc cremoris	NCDO 543	positive	+ve Probiotic/Food Production
Micrococcus luteus	NCIB 8165	positive	Commensal
Moraxella sp.	NCIB 10762	negative	Food Spoilage
Proteus vulgaris	NCIB 4175	negative	Food Spoilage
Pseudomonas aeruginosa	NCIB 950	negative	Food Spoilage/Human Pathogen
Salmonella pullorum	NCTC 10704	negative	Food Poisoning - Poultry.
Serratia marcescens	NCIB 1377	negative	Commensal/2° Pathogen
Staphylococcus aureus	NCIB 6571	positive	Food Poisoning
Yersinia enterocolitica	NCTC 10460	negative	Food Poisoning

[a] NCIB, National Collection of Industrial Bacteria; NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; NCPPB, National Collection of Plant Pathogenic Bacteria; NCDO, National Collection of Dairy Organisms.

3.3. Results

3.3.1. Antibacterial Activity Screening of the Volatile Oils from Black Pepper, Clove, Geranium, Melissa, Nutmeg, Oregano and Thyme

The antibacterial activities of the plant volatile oils of black pepper, clove, geranium, melissa, nutmeg, oregano and thyme are shown in Table 24 and Table 25. All the volatile oils demonstrated some degree of antibacterial activity.

3.3.1.1. Black Pepper

The antibacterial evaluation of the volatile oil from black pepper is shown in Table 24. With the exception of *E. faecalis*, *K. pneumoniae* and *L. plantarum*, all the remaining organisms demonstrated some degree of sensitivity to this volatile oil. The order of decreasing sensitivity was found to be *A. hydrophila* > *L. cremoris* > *B. linens* > *S. aureus* > *E. carotovora* > *M. luteus* > *A. calcoacetica* > *C. freundii* > *Y. enterocolitica* > *B. natriegens* > *F. suaveolens* > *B. subtilis* > *E. aerogenes* > *C. sporogenes* > *P. aeruginosa* > *S. marcescens* > *E. coli* > *B. thermosphacta* > *S. pullorum* > *P. vulgaris* > *A. faecalis* > *Moraxella sp.*. Growth of *A. hydrophila* was completely inhibited by 15μL of undiluted oil. *Moraxella sp.* was only marginally affected.

Black pepper was more effective against *B. natriegens* than the antibiotics used in this study, while in the case of *E. coli*, only the antibiotic novobiocin was more effective than this oil, Table 23.

3.3.1.2. Clove

The antibacterial evaluation of the volatile oil from clove is shown in Table 24. Clove volatile oil demonstrated activity against all the test organisms but was unable to inhibit completely the growth of the test bacteria. The order of decreasing sensitivity was found to be *B. linens* > *L. plantarum* > *A. faecalis* > *S. marcescens* > *B. subtilis* > *L. cremoris* > *B. natriegens* ~ *Moraxella sp.* > *E. aerogenes* > *S. aureus* > *M. luteus* > *F. suaveolens* > *P. aeruginosa* ~ *S. pullorum* > *Y. enterocolitica* > *E. coli* > *C. sporogenes* > *A. hydrophila* ~ *E. carotovora* > *B. thermosphacta* > *A. calcoacetica* > *K. pneumoniae* ~ *P. vulgaris* > *E. faecalis*. The most resistant

organisms were *E. faecalis* (7.8±1.1mm), *K. pneumoniae* (9.1±0.0mm) and *P. vulgaris* (9.1±0.6mm). The organisms appeared to fall within 4 inhibition ranges: 29.2±0.7mm (n=2), 21.1±0.9mm (n=4), 14.5±0.2mm (n=12) and 10.5±0.5mm (n=6).

Clove volatile oil was more effective than the antibiotics used in this study against the microorganisms *A. faecalis*, *B. natriegens*, *B. linens* except for the antibiotic neomycin; *C. freundii* except for the antibiotics tetracycline and ampicillin; *E. aerogenes*, *E. coli* except for the antibiotic novobiocin and *L. plantarum*. Against the microorganisms *M. luteus*, *Moraxella* sp. and *S. marcescens* it was as effective as the antibiotics erythromycin, oleandomycin and tetracycline, Table 23.

3.3.1.3. Geranium

The antibacterial evaluation of the volatile oil from geranium is shown in Table 24. Geranium volatile oil demonstrated antimicrobial activity against the majority of organisms tested. The order of decreasing sensitivities being *F. suaveolens* > *E. aerogenes* > *P. aeruginosa* > *L. cremoris* > *C. freundii* > *K. pneumoniae* > *S. aureus* > *M. luteus* > *A. calcoacetica* > *B. subtilis* > *B. natriegens* > *B. thermosphacta* > *S. marcescens* > *C. sporogenes* > *B. linens* > *S. pullorum.* The remaining organisms *A. hydrophila*, *A. faecalis*, *E. faecalis*, *E. carotovora*, *E. coli*, *L. plantarum*, *Moraxella sp.*, *P. vulgaris* and *Y. enterocolitica* were all resistant to any biocidal effects, successfully growing without demonstrating any inhibition of growth. Interestingly, the pathogen *E. faecalis* and the soil organism *L. plantarum* were the only Gram positive bacteria resistant to the effects of this oil. The Gram negative strains *F. suaveolens* (30.9±5.4mm), *E. aerogenes* (19.8±2.1mm), *P. aeruginosa* (19.4±0.1mm), *C. freundii* (16.0±2.0mm), *K. pneumoniae* (13.8±0.2mm) and *A. calcoacetica* (13.0±0.3mm) appeared to be the most sensitive.

Geranium essential oil was more effective than the antibiotics used in this study against the microorganisms *B. natriegens*, *C. freundii* except for the antibiotic ampicillin, *E. aerogenes*, *F. suaveolens* and *P. aeruginosa*, Table 23.

3.3.1.4. Melissa

The antibacterial evaluation of the volatile oil from melissa is shown in Table 25. In the melissa study, *A. faecalis*, *E. carotovora*, *E. faecalis*, *K. pneumoniae*, *Moraxella sp.*, *P. aeruginosa*, *P. vulgaris*, *S. marcescens* and *S. pullorum* were all

resistant to any possible inhibitory effects 15μL of undiluted oil possessed. The oil demonstrated activity against *A. calcoacetica*, *B. linens*, *B. natriegens*, *B. subtilis*, *B. thermosphacta*, *E. coli*, *F. suaveolens*, *L. plantarum*, *M. luteus*, *S. aureus* and *Y. enterocolitica*. The most sensitive organisms were *B. subtilis* [54.9±1.9mm] and *L. plantarum* [36.0±0.7mm]. There appears to be a distinct difference between bacterial type and sensitivity to the plant volatile oil of melissa. All the resistant bacteria, with the exception of *E. faecalis*, were Gram -ve and the most sensitive are Gram +ve. This trend would suggest that melissa and its components may act at the cell wall/membrane.

Melissa volatile oil was more effective than the antibiotics used in this study against the microorganisms *B. natriegens*, *B. subtilis*, *E. coli* and *F. suaveolens* except for the antibiotic novobiocin, *K. pneumoniae* except for the antibiotic erythromycin and *S. aureus* except for the antibiotic ampicillin, Table 23.

Table 23. Zones of growth inhibition (mm) for a variety of antibiotics, well diameter of 4.0mm.

Bacterial Strain	Antibiotic[a]									
	C10	E10	SF100	NV5	OL5	P1.5	S10	Te10	N10	A10
Acinetobacter calcoacetica	4.0±0.0	16.1±0.5	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	22.2±0.7	4.0±0.0	15.6±2.0	8.2±1.0
Aeromones hydrophila	20.2±0.8	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	17.0±1.2	12.8±0.5	4.0±0.0
Alcaligenes faecalis	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	15.9±1.0	4.0±0.0
Bacillus subtilis	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	13.7±1.2	8.3±0.2
Beneckea natriegens	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	10.3±0.8	9.7±0.0
Brevibacterium linens	18.6±0.7	25.7±0.9	15.6±1.2	17.0±1.5	15.0±1.2	22.0±4.2	22.8±0.4	18.7±2.1	50.8±2.7	23.9±1.2
Brocothrix thermosphacta	15.0±0.6	18.0±0.2	23.3±1.5	15.4±2.3	15.5±3.0	4.0±0.0	13.9±0.5	13.8±0.9	14.8±0.9	22.3±1.0
Citrobacter freundii	12.5±0.5	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	14.0±0.6	14.4±0.8	12.0±1.0	17.0±0.8
Clostridium sporogenes	33.5±1.3	39.2±2.0	21.6±0.9	4.0±0.0	29.6±1.5	38.0±1.4	20.8±1.2	40.0±4.9	16.1±0.4	55.4±2.4
Enterobacter aerogenes	14.4±0.6	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	12.2±1.1	14.3±0.5	9.7±0.7	4.0±0.0
Enterococcus faecalis	13.6±0.7	4.0±0.0	21.7±0.9	4.0±0.0	4.0±0.0	4.0±0.0	14.3±2.4	12.8±0.6	14.0±0.4	31.0±0.6
Erwinia carotovora	14.0±0.6	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	15.5±2.0	14.7±1.0	18.6±0.6	18.7±0.7
Escherichia coli	4.0±0.0	4.0±0.0	4.0±0.0	14.7±0.9	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Flavobacterium suaveolens	4.0±0.0	11.0±0.3	4.0±0.0	26.0±1.0	12.0±0.5	4.0±0.0	15.0±0.6	4.0±0.0	4.0±0.0	7.6±0.4
Klebsiella pneumoniae	12.6±0.6	22.0±0.4	4.0±0.0	4.0±0.0	15.4±0.4	4.0±0.0	12.2±0.4	14.4±0.4	15.8±0.8	14.9±0.8
Lactobacillus plantarum	11.6±0.5	15.0±0.0	4.0±0.0	4.0±0.0	18.8±1.8	4.0±0.0	12.5±1.0	19.0±0.9	12.7±0.4	12.5±0.1
Leuconostoc cremoris	14.2±0.6	18.5±0.8	19.0±0.6	18.4±0.9	14.0±2.0	14.2±1.9	14.6±2.2	13.9±0.2	18.9±0.2	31.9±0.9
Micrococcus luteus	26.0±1.0	14.8±1.0	4.0±0.0	16.9±1.5	14.8±1.4	12.2±2.0	24.2±2.1	14.8±0.8	26.0±0.2	37.4±1.0
Moraxella sp.	14.6±0.4	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	12.1±0.4	4.0±0.0	11.9±0.3	11.0±0.9
Proteus vulgaris	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	14.1±0.9	12.1±1.0	16.3±0.5	4.0±0.0

Table 23 *continued.* Zones of growth inhibition (mm) for a variety of antibiotics, well diameter of 4.0mm.

Bacterial Strain	Antibiotic[a]									
	C10	E10	SF100	NV5	OL5	P1.5	S10	Te10	N10	A10
Pseudomonas aeruginosa	12.5±0.6	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	10.5±0.9	12.0±0.6	15.0±0.5	16.9±0.9
Salmonella pullorum	13.2±0.8	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	23.9±0.5	4.0±0.0	18.7±0.9	7.6±0.5
Serratia marcescens	15.7±0.4	16.6±0.4	4.0±0.0	21.0±2.6	17.9±1.6	4.0±0.0	12.1±1.8	4.0±0.0	15.0±1.0	9.8±0.8
Staphylococcus aureus	15.8±0.5	14.1±0.6	4.0±0.0	14.1±2.0	4.0±0.0	4.0±0.0	4.0±0.0	19.0±2.0	4.0±0.0	26.1±1.0
Yersinia enterocolitica	13.6±0.9	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	14.3±1.0	15.5±2.3	16.5±1.0	18.3±0.8

All values are represented as the mean (mm) ± SEM for duplicate experiments. n.d.: not determined. 4.0: no effect. *: no bacterial growth. [a] C10, Chloramphenicol; E10, Erythromycin; SF100, Sulphafuragole; NV5, Novobiocin; OL5, Oleandomycin; P1.5, Penicillin; S10, Streptomycin; Te10, Tetracycline; N10, Neomycin; A10, Ampicillin.

Table 24. Zones of growth inhibition (mm) showing antibacterial activity for a number of selected plant volatile oils, well diameter of 4.0mm.

Bacterial Strains	Volatile Oil		
	Black Pepper	Clove	Geranium
Acinetobacter calcoacetica	12.3±2.0	10.3±0.2	13.0±0.3
Aeromones hydrophila	*	11.7±1.1	4.0±0.0
Alcaligenes faecalis	7.1±0.8	23.1±0.6	4.0±0.0
Bacillus subtilis	9.5±0.6	21.1±0.0	11.4±0.6
Beneckea natriegens	10.8±0.7	15.8±0.7	11.0±0.7
Brevibacterium linens	15.9±1.0	29.8±0.1	7.6±0.1
Brocothrix thermosphacta	7.2±0.0	11.1±0.1	8.6±0.5
Citrobacter freundii	12.0±1.6	14.1±2.6	16.0±2.0
Clostridium sporogenes	8.7±0.3	13.4±0.5	7.8±0.6
Enterobacter aerogenes	8.8±0.9	15.5±0.6	19.8±2.1
Enterococcus faecalis	4.0±0.0	7.8±1.1	4.0±0.0
Erwinia carotovora	12.9±1.0	11.7±0.4	4.0±0.0
Escherichia coli	7.3±0.4	13.6±0.3	4.0±0.0
Flavobacterium suaveolens	10.1±0.1	14.4±0.2	30.9±5.4
Klebsiella pneumoniae	4.0±0.0	9.1±0.0	13.8±0.2
Lactobacillus plantarum	4.0±0.0	28.5±1.0	4.0±0.0
Leuconostoc cremoris	16.3±0.8	18.7±0.6	16.7±2.3
Micrococcus luteus	12.4±0.0	14.8±0.8	13.3±0.4
Moraxella sp.	5.4±0.2	15.8±0.8	4.0±0.0
Proteus vulgaris	7.1±0.3	9.1±0.6	4.0±0.0
Pseudomonas aeruginosa	7.7±0.9	14.0±1.9	19.4±0.1
Salmonella pullorum	7.1±0.2	14.0±0.8	6.9±0.6
Serratia marcescens	7.5±0.4	21.6±0.9	8.5±0.4
Staphylococcus aureus	14.5±0.3	14.9±0.1	13.6±0.3
Yersinia enterocolitica	11.7±2.2	13.7±0.1	4.0±0.0

All values are represented as the mean (mm) ± SEM for duplicate experiments. n.d.: not determined. 4.0: no effect. *: no bacterial growth.

Table 25. Zones of growth inhibition (mm) showing antibacterial activity for a number of selected plant volatile oils, well diameter of 4.0mm.

Bacterial Strains	Volatile Oil			
	Melissa	Nutmeg	Oregano	Thyme
Acinetobacter calcoacetica	10.7±0.5	12.7±1.3	52.2±1.5	30.7±0.5
Aeromones hydrophila	n.d.	*	*	*
Alcaligenes faecalis	4.0±0.0	9.0±0.3	33.6±0.1	53.8±1.2
Bacillus subtilis	54.9±1.9	7.0±0.4	20.5±0.4	23.4±1.2
Beneckea natriegens	17.1±2.4	10.0±1.2	37.1±3.2	*
Brevibacterium linens	9.8±1.1	22.2±0.3	*	*
Brocothrix thermosphacta	9.3±0.6	9.7±0.7	31.2±0.8	*
Citrobacter freundii	n.d.	12.8±0.0	29.6±0.8	*
Clostridium sporogenes	n.d.	4.0±0.0	*	*
Enterobacter aerogenes	4.0±0.0	18.5±1.2	17.9±0.8	41.8±0.8
Enterococcus faecalis	4.0±0.0	4.0±0.0	14.6±0.1	15.2±0.7
Erwinia carotovora	4.0±0.0	14.1±2.6	31.2±1.4	35.8±4.4
Escherichia coli	11.6±1.0	10.4±0.0	29.5±3.4	32.4±0.1
Flavobacterium suaveolens	13.8±0.2	16.9±0.9	9.4±0.7	*
Klebsiella pneumoniae	4.0±0.0	16.9±0.9	19.0±1.5	31.8±0.5
Lactobacillus plantarum	36.0±0.7	4.0±0.0	23.8±0.3	26.3±0.4
Leuconostoc cremoris	n.d.	4.0±0.0	*	*
Micrococcus luteus	7.2±0.9	11.7±0.3	21.5±0.1	*
Moraxella sp.	4.0±0.0	6.4±0.2	31.4±1.9	29.0±5.6
Proteus vulgaris	4.0±0.0	10.0±1.1	44.6±4.9	*
Pseudomonas aeruginosa	4.0±0.0	4.0±0.0	*	33.5±2.0
Salmonella pullorum	4.0±0.0	8.4±0.5	46.0±6.7	*
Serratia marcescens	4.0±0.0	8.2±0.3	18.9±0.4	39.1±0.8
Staphylococcus aureus	11.2±0.3	24.6±0.4	17.6±0.5	*
Yersinia enterocolitica	11.7±0.4	7.3±0.4	33.9±0.4	25.5±2.9

All values are represented as the mean (mm) ± SEM for duplicate experiments. n.d.: not determined. 4.0: no effect. *: no bacterial growth.

3.3.1.5. Nutmeg

The antibacterial evaluation of the volatile oil from nutmeg is shown in Table 25. The test organisms decreasing order of sensitivity was found to be *A. hydrophila* > *S. aureus* > *B. linens* > *E. aerogenes* > *K. pneumoniae* > *E. carotovora* > *C. freundii* > *A. calcoacetica* > *M. luteus* > *E. coli* > *B. natriegens* ~ *P. vulgaris* > *B. thermosphacta* > *A. faecalis* > *S. pullorum* > *S. marcescens* > *Y. enterocolitica* > *B. subtilis* > *Moraxella sp.* > *C. sporogenes* > *L. plantarum* >*L. cremoris* > *P. aeruginosa* > *E. faecalis* > *F. suaveolens*. The most sensitive organism was *A. hydrophila*. Nutmeg completely prevented this culture from growing. *C. sporogenes*, *L. plantarum*, *L. cremoris*, *P. aeruginosa* and *E. faecalis* were found to be completely resistant to any effects this plant volatile oil possessed.

Nutmeg volatile oil was more effective than the antibiotics used in this study against the microorganisms *A. hydrophila*, *A. faecalis* except for the antibiotic neomycin, *E. aerogenes*, *E. coli* except for the antibiotic novobiocin, *F. suaveolens* except for the antibiotic novobiocin, *K. pneumoniae* except for the antibiotic erythromycin and *S. aureus* except for the antibiotic ampicillin, Table 23.

3.3.1.6. Oregano

Oregano volatile was shown to have antimicrobial activity against all the bacterial strains tested, Table 25. The microorganisms *A. hydrophila*, *B. linens*, *C. sporogenes*, *L. cremoris* and *P. aeruginosa* were completely inhibited. The decreasing order of sensitivity of the remaining organisms were shown to be *A. calcoacetica* > *P. vulgaris* > *B. natriegens* > *Y. enterocolitica* > *A. faecalis* > *Moraxella sp.* > *B. thermosphacta* ~ *E. carotovora* > *C. freundii* > *E. coli* > *L. plantarum* > *M. luteus* > *B. subtilis* > *K. pneumoniae* > *S. marcescens* > *E. aerogenes* > *S. aureus* > *E. faecalis* > *F. suaveolens*.

The plant volatile oil of oregano was more effective than the antibiotics used in this against the microorganisms *B. thermosphacta* except for the antibiotic neomycin; *E. faecalis* except for the antibiotics sulphafuragole and ampicillin; *K. pneumoniae* except for the antibiotic erythromycin; *S. marcescens* except for the antibiotic novobiocin and *S. aureus* except for the antibiotics tetracycline and ampicillin, Table 23.

3.3.1.7. Thyme

Thyme volatile exhibited the most potent biocidal activity, completely inhibiting the growth of *A. hydrophila*, *B. natriegens*, *B. thermosphacta*, *C. freundii*, *C. sporogenes*, *F. suaveolens*, *L. cremoris*, *M. luteus*, *P. vulgaris*, *S. aureus* and *S. pullorum*, Table 25. The order of decreasing sensitivity of the remaining organisms was found to be *A. faecalis* > *E. aerogenes* > *S. marcescens* > *E. carotovora* > *P. aeruginosa* > *E. coli* > *K. pneumoniae* > *A. calcoacetica* > *Moraxella sp.* > *L. plantarum* > *Y. enterocolitica* > *B. subtilis* > *E. faecalis*. The Gram -ve organisms which did grow were the most sensitive to thyme, with the exception of *Y. enterocolitica* (25.5±2.9mm) which was less sensitive than the Gram +ve *L. plantarum* bacterium (26.3±0.4mm). The Gram -ve bacteria were the most resistant.

The plant volatile oil of thyme was more effective than all the antibiotics used in this study except in the case of *E. faecalis* in which case the antibiotics sulphafuragole and ampicillin were more effective, Table 23.

3.3.2. Antibacterial Activity Screening of the Main Components

The analysis of the chemical composition of the plant volatile oils used in this study confirm that plant volatile oils are complex and varied mixtures rich in principally terpenes (Chapter 2). These components vary in primary structure (e.g. saturated and unsaturated acyclic, cyclic and heterocyclic structures) with varied functional moieties including acetates, alcohols, aldehydes, formates, ketones, methoxyl groups, tiglates and phenolic groups. Consequently, the activity of the oils would be expected to relate to:

1. The respective composition of the plant volatile oils.
2. The component's structural configuration and their functional groups.
3. Synergistic interactions between the oil components.

The aims of this present investigation were as follows:

1. To assess the antibacterial activities of a number of components determined to be present in the plant volatile oils tested for antibacterial properties.
2. Determine which components are the most active.

3. Explain the activity the components in terms of their structure.

Aromadendrene

Aromadendrene, a constituent of black pepper [0.47%] (Table 10), geranium [0.41%] (Table 12) and nutmeg [Serva] [0.14%] (Table 19) volatile oils, was unable to inhibit the growth of the test organisms, Table 26.

α-Bisabolol

α-Bisabolol demonstrated limited activity against the microorganisms in this study, Table 26. Five organisms were marginally inhibited from growth, with decreasing sensitivity being *E. faecalis* > *C. sporogenes* > *F. suaveolens* > *B. linens* > *S. marcescens*.

Borneol

Borneol, a constituent of monarda [0.27%] (Table 17), nutmeg [0.25%] (Table 18), nutmeg (Serva) [0.34%] (Table 19), oregano [0.43%] (Table 20), thyme [0.86%] (Table 21) volatile oils demonstrated some degree of antibacterial activity, Table 26. Less than half the tested organisms demonstrated a degree of growth inhibition. Their decreasing sensitivity were *B. subtilis* > *B. natriegens* > *A. hydrophila* > *B. thermosphacta* > *A. calcoacetica* ~ *F. suaveolens* > *S. aureus* > *B. linens* > *E. coli* > *S. marcescens*. The antibacterial activity of borneol was in general agreement with previously reported data (Deans and Svoboda, 1988; Deans and Svoboda, 1989; Morris *et al.,* 1979).

Borneol was more effective than the antibiotics used in this study against the microorganisms *A. calcoacetica* except for the antibiotics erythromycin, streptomycin, neomycin and ampicillin; *A. hydrophila* except for the antibiotics chloramphenicol, tetracycline and neomycin; *B. subtilis* and *B. natriegens* except for the antibiotics neomycin and ampicillin; *E. coli* except for the antibiotic novobiocin and *F. suaveolens* except for the antibiotics erythromycin, novobiocin, oleandomycin, streptomycin and ampicillin, Table 23.

Bornyl Acetate

Bornyl acetate, a constituent of the volatile oils of lovage stem [0.67%] (Table 15), nutmeg [0.11%] (Table 18), oregano [0.11%] (Table 20) and thyme [1.91%] (Table 21) demonstrated poor antibacterial activity. This component only

marginally affected the growth of *A. calcoacetica*, *A. hydrophila*, *B. linens*, *B. thermosphacta*, *M. luteus* and *S. aureus*. The order of sensitivity being *M. luteus* > *A. calcoacetica* > *B. linens* > *B. thermosphacta* > *A. hydrophila* > *S. aureus*, Table 26.

Bornyl acetate was more effective than the antibiotics used in this study against the microorganisms *A. calcoacetica* except for the antibiotics erythromycin, streptomycin and neomycin and *A. hydrophila* except for the antibiotics chloramphenicol, tetracycline and neomycin, Table 23.

δ-3-Carene

δ-3-Carene, a constituent of the volatile oils of black pepper [8.53%] (Table 10), nutmeg [0.89%] (Table 18) and oregano [0.14%] (Table 20) demonstrated variable degrees of growth inhibition against all the test organisms, Table 27. The microorganisms *B. thermosphacta*, *C. freundii* and *L. plantarum* were completely inhibited from growing after a 48h incubation period. The order of decreasing sensitivity was found to be *C. sporogenes* > *Y. enterocolitica* > *A. faecalis* > *S. pullorum* > *E. coli* > *Moraxella sp.* > *E. faecalis* > *K. pneumoniae* > *E. aerogenes* ~ *S. aureus* > *A. hydrophila* ~ *M. luteus* ~ *P. vulgaris* > *F. suaveolens* > *P. aeruginosa* > *A. calcoacetica* > *B. subtilis* > *B. linens* > *S. marcescens*.

δ-3-carene was more effective than the antibiotics used in this study against the microorganisms *A. calcoacetica* except for the antibiotics erythromycin, streptomycin and neomycin; *A. hydrophila* except for the antibiotics chloramphenicol, tetracycline and neomycin; *A. faecalis* except for the antibiotic neomycin; *B. subtilis* except for the antibiotics neomycin and ampicillin; *E. aerogenes* except for the antibiotics chloramphenicol, streptomycin, tetracycline and neomycin; *E. coli* except for the antibiotic novobiocin; *Moraxella sp.* except for chloramphenicol; *P. vulgaris* except for the antibiotics streptomycin, tetracycline and neomycin; *P. aeruginosa* except for chloramphenicol, tetracycline, neomycin and ampicillin; *S. pullorum* except for the antibiotics neomycin and ampicillin and *Y. enterocolitica* except for the antibiotics tetracycline, neomycin and ampicillin, Table 23.

Carvacrol

Carvacrol, a constituent of the volatile oils of monarda [6.12%] (Table 17), oregano [69.37%] (Table 20) and thyme [2.95%] (Table 21) was very potent at

inhibiting the growth of all the test organisms, as shown in Table 27. The order of sensitivity was found to be *A. calcoacetica* > *B. subtilis* > *A. hydrophila* > *E. coli* > *S. pullorum* > *M. luteus* > *P. vulgaris F. suaveolens* ~ *P. aeruginosa* > *B. thermosphacta* > *K. pneumoniae* > *S. marcescens* > *Y. enterocolitica* > *A. faecalis* > *B. linens* > *Moraxella sp.* > *E. aerogenes* > *C. sporogenes* > *S. aureus* > *L. plantarum* > *E. faecalis* > *C. freundii* > E. *carotovora* > *B. natriegens*.

Carvacrol was more effective than the antibiotics used in this study against the microorganisms *A. calcoacetica*, *A. hydrophila*, *A. faecalis*, *B. subtilis*, *B. natriegens*, *B. thermosphacta*, *C. freundii*, *E. aerogenes*, *E. coli*, *K. pneumoniae*, *L. plantarum*, *Moraxella sp.*, *P. vulgaris*, *P. aeruginosa*, *S. pullorum*, *S. marcescens*, *S. aureus* and *Y. enterocolitica*. In the case of the organisms *E. faecalis* and *E. carotovora*, the antibiotics sulphafuragole and ampicillin and neomycin and ampicillin were more effective, respectively, while in the case of *F. suaveolens* and *M. luteus*, the antibiotics novobiocin and ampicillin were more effective, respectively, Table 23.

Carvacrol Methyl Ether

Carvacrol methyl ether, a constituent of the volatile oil of monarda [0.69%] (Table 17) demonstrated minimal inhibitory properties against the test organisms inhibiting 8 of 25 organisms, as shown in Table 27. Carvacrol methyl ether was marginally more effective against the microorganism *A. faecalis*, *B. natriegens*, *C. sporogenes*, *F. suaveolens*, *K. pneumoniae*, *L. plantarum*, *P. vulgaris* and *S. pullorum*, which were all resistant to the antibiotics used in this study. Only the antibiotic novobiocin was more effective against *E. coli* than this volatile oil component, Table 23.

β-Caryophyllene

β-Caryophyllene, a constituent of the volatile oils of black pepper [24.21%] (Table 10), clove [5.58%] (Table 11), geranium [1.20%] (Table 12), monarda [0.64%] (Table 17), nutmeg [Serva] [4.83%] (Table 19) and thyme [0.98%] (Table 21) demonstrated activity against 6/24 bacteria, as shown in Table 27. The degree of sensitivity being *C. sporogenes* > *B. subtilis* > *B. natriegens* > *C. freundii* > *S. marcescens* > *B. linens*.

β-Caryophyllene was marginally more effective against the microorganisms *B. subtilus* and *B. natriegens* than the antibiotics used in this study, except for the antibiotics neomycin and ampicillin, Table 23.

1,8-Cineole

1,8-Cineole, a constituent of the volatile oils of monarda [0.26%] (Table 17), oregano [0.45%] (Table 20) and thyme [1.25%] (Table 21) demonstrated limited activity as shown in Table 28.

1,8-Cineole was more effective than the antibiotics used in this study against the mircoorganisms *A. faecalis*, except for the antibiotic neomycin, and *B. natriegens*, except for the antibiotics neomycin and ampicillin, Table 23.

Citral (cis+trans)

Citral (*cis+trans*) demonstrated activity against all the test bacteria, as shown in Table 28. The order of sensitivity of the organisms was found to be *E. aerogenes* > *C. sporogenes* > *S. pullorum* > *E. coli* > *E. carotovora* > *Y. enterocolitica* > *K. pneumoniae* > *A. faecalis* > *A. calcoacetica* ~ *A. hydrophila* ~ *L. plantarum* > *B. linens* > *B. natriegens* > *C. freundii* ~ *P. vulgaris* > *M. luteus* > *F. suaveolens* ~ *P. aeruginosa* > *Moraxella sp.* > *E. faecalis* > *B. thermosphacta* > *S. marcescens* > *B. subtilis* > *S. aureus*.

Citral was marginally more effective than the antibiotics used in this study in the case of the microorganism *E. aerogenes* while in the cases of *E. coli* and *S. pullorum*, *E. coli* was more sensitive to the antibiotic novobiocin while *S. pullorum* was more sensitive to the antibiotics chloramphenicol, streptomycin and neomycin, Table 23.

Table 26. Zones of growth inhibition (mm) showing antibacterial activity for a number phytoconstituents from selected plant volatile oils, well diameter of 4.0mm.

Bacterial Strains	Volatile Oil Components			
	Aromadendrene	α-Bisabolol	Borneol	Bornyl Acetate
Acinetobacter calcoacetica	4.0±0.0	4.0±0.0	7.0±1.1	8.6±0.6
Aeromones hydrophila	4.0±0.0	4.0±0.0	8.2±0.5	7.3±0.6
Alcaligenes faecalis	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Bacillus subtilis	4.0±0.0	4.0±0.0	10.4±0.5	4.0±0.0
Beneckea natriegens	4.0±0.0	4.0±0.0	9.1±0.5	4.0±0.0
Brevibacterium linens	4.0±0.0	6.5±0.2	6.7±0.4	8.4±0.7
Brocothrix thermosphacta	4.0±0.0	4.0±0.0	7.4±0.4	7.4±0.3
Citrobacter freundii	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Clostridium sporogenes	6.0±0.1	7.0±0.3	4.0±0.0	n.d.
Enterobacter aerogenes	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Enterococcus faecalis	4.0±0.0	7.9±0.5	4.0±0.0	4.0±0.0
Erwinia carotovora	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Escherichia coli	4.0±0.0	4.0±0.0	6.7±0.3	4.0±0.0
Flavobacterium suaveolens	4.0±0.0	6.6±0.2	7.0±0.0	4.0±0.0
Klebsiella pneumoniae	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Lactobacillus plantarum	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Leuconostoc cremoris	n.d.	n.d.	n.d.	n.d.
Micrococcus luteus	4.0±0.0	4.0±0.0	4.0±0.0	8.8±0.8
Moraxella sp.	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Proteus vulgaris	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Pseudomonas aeruginosa	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Salmonella pullorum	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Serratia marcescens	4.0±0.0	5.6±0.5	5.4±0.6	4.0±0.0
Staphylococcus aureus	4.0±0.0	4.0±0.0	6.9±0.2	6.9±0.4
Yersinia enterocolitica	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0

All values are represented as the mean (mm) ± SEM for duplicate experiments. n.d.: not determined. 4.0: no effect. *: no bacterial growth.

Table 27. Zones of growth inhibition (mm) showing antibacterial activity for a number phytoconstituents from selected plant volatile oils, well diameter of 4.0mm.

Bacterial Strains	Volatile Oil Components			
	δ-3-Carene	Carvacrol	Carvacrol Methyl Ether	β-Caryophyllene
Acinetobacter calcoacetica	10.0±0.2	45.3±1.3	4.0±0.0	4.0±0.0
Aeromones hydrophila	11.1±0.8	37.7±2.4	4.0±0.0	4.0±0.0
Alcaligenes faecalis	14.4±0.7	21.8±1.6	5.9±0.7	4.0±0.0
Bacillus subtilis	9.5±0.3	39.5±1.0	4.0±0.0	8.0±0.2
Beneckea natriegens	±	14.1±0.3	6.8±0.3	6.8±0.8
Brevibacterium linens	9.1±0.5	21.7±0.5	4.0±0.0	5.7±0.3
Brocothrix thermosphacta	4.0±0.0	25.5±1.0	4.0±0.0	4.0±0.0
Citrobacter freundii	4.0±0.0	17.7±0.1	4.0±0.0	6.1±0.1
Clostridium sporogenes	16.6±0.5	20.3±0.7	8.9±0.5	11.3±1.4
Enterobacter aerogenes	11.3±0.7	21.2±0.4	4.0±0.0	4.0±0.0
Enterococcus faecalis	12.0±0.8	18.5±0.8	4.0±0.0	4.0±0.0
Erwinia carotovora	11.0±0.4	15.5±0.7	4.0±0.0	4.0±0.0
Escherichia coli	13.5±1.2	29.2±0.2	5.9±0.6	4.0±0.0
Flavobacterium suaveolens	10.9±0.4	26.0±1.8	5.1±0.5	4.0±0.0
Klebsiella pneumoniae	11.7±0.6	23.6±0.0	7.1±0.3	4.0±0.0
Lactobacillus plantarum	4.0±0.0	18.7±0.7	6.3±0.7	4.0±0.0
Leuconostoc cremoris	n.d.	n.d.	n.d.	n.d.
Micrococcus luteus	11.1±0.4	26.6±0.5	4.0±0.0	4.0±0.0
Moraxella sp.	12.3±0.7	21.6±0.0	4.0±0.0	4.0±0.0
Proteus vulgaris	11.1±0.4	26.5±1.6	6.2±0.1	4.0±0.0
Pseudomonas aeruginosa	10.6±2.0	26.0±0.4	4.0±0.0	4.0±0.0
Salmonella pullorum	13.8±0.8	27.1±0.7	5.0±0.5	4.0±0.0
Serratia marcescens	8.0±1.2	22.5±0.9	4.0±0.0	6.0±0.3
Staphylococcus aureus	11.3±0.6	20.2±0.5	4.0±0.0	4.0±0.0
Yersinia enterocolitica	15.4±0.2	22.4±1.2	4.0±0.0	4.0±0.0

All values are represented as the mean (mm) ± SEM for duplicate experiments. n.d.: not determined. 4.0: no effect. *: no bacterial growth.

Table 28. Zones of growth inhibition (mm) showing antibacterial activity for a number phytoconstituents from selected plant volatile oils, well diameter of 4.0mm.

Bacterial Strains	Volatile Oil Components			
	1,8-Cineole	Citral (*cis+trans*)	Citronellal	*p*-Cymene
Acinetobacter calcoacetica	10.9±1.0	7.9±0.9	4.0±0.0	4.0±0.0
Aeromones hydrophila	4.0±0.0	7.9±0.3	4.0±0.0	4.0±0.0
Alcaligenes faecalis	6.9±0.9	8.4±0.4	4.0±0.0	4.0±0.0
Bacillus subtilis	4.0±0.0	5.9±0.3	11.6±0.2	4.0±0.0
Beneckea natriegens	5.8±1.1	7.3±0.7	4.0±0.0	4.0±0.0
Brevibacterium linens	4.0±0.0	7.5±0.3	4.0±0.0	4.0±0.0
Brocothrix thermosphacta	8.3±0.4	6.1±0.1	4.0±0.0	4.0±0.0
Citrobacter freundii	4.0±0.0	6.9±1.0	4.0±0.0	4.0±0.0
Clostridium sporogenes	6.45±0.4	12.6±0.2	10.7±0.3	4.0±0.0
Enterobacter aerogenes	4.0±0.0	21.6±0.1	4.0±0.0	4.0±0.0
Enterococcus faecalis	4.0±0.0	6.2±0.7	5.6±0.5	4.0±0.0
Erwinia carotovora	4.0±0.0	10.2±0.1	4.0±0.0	4.0±0.0
Escherichia coli	4.0±0.0	11.0±0.2	4.0±0.0	4.0±0.0
Flavobacterium suaveolens	4.0±0.0	6.6±0.1	8.3±0.3	4.0±0.0
Klebsiella pneumoniae	4.0±0.0	8.8±0.2	4.0±0.0	4.0±0.0
Lactobacillus plantarum	4.75±0.3	7.9±0.4	4.0±0.0	4.0±0.0
Leuconostoc cremoris	n.d.	n.d.	n.d.	n.d.
Micrococcus luteus	4.0±0.0	6.7±0.4	8.1±0.8	4.0±0.0
Moraxella sp.	10.2±0.5	6.5±0.4	4.0±0.0	4.0±0.0
Proteus vulgaris	4.0±0.0	6.9±0.1	4.0±0.0	4.0±0.0
Pseudomonas aeruginosa	4.0±0.0	6.6±0.0	7.3±0.2	4.0±0.0
Salmonella pullorum	4.0±0.0	12.0±0.1	4.0±0.0	4.0±0.0
Serratia marcescens	6.0±1.0	6.0±0.6	4.0±0.0	4.0±0.0
Staphylococcus aureus	4.0±0.0	4.9±0.1	4.0±0.0	4.0±0.0
Yersinia enterocolitica	4.0±0.0	9.0±0.6	4.0±0.0	4.0±0.0

All values are represented as the mean (mm) ± SEM for duplicate experiments. n.d.: not determined. 4.0: no effect. *: no bacterial growth.

Table 29. Zones of growth inhibition (mm) showing antibacterial activity for a number phytoconstituents from selected plant volatile oils, well diameter of 4.0mm.

Bacterial Strains	Volatile Oil Components			
	Eugenol	Geraniol	Geranyl Acetate	*cis*-Hex-3-an-1-ol
Acinetobacter calcoacetica	15.4±0.3	6.1±0.2	10.3±0.3	8.1±0.1
Aeromones hydrophila	17.0±0.4	6.4±0.5	9.0±0.4	8.5±0.3
Alcaligenes faecalis	12.3±0.5	7.0±0.2	10.5±0.1	9.3±0.6
Bacillus subtilis	21.8±0.4	6.4±0.6	10.8±0.2	6.4±0.7
Beneckea natriegens	20.8±1.8	6.2±0.4	10.8±0.1	7.6±0.5
Brevibacterium linens	12.7±0.1	7.3±0.6	12.5±0.8	8.1±0.2
Brocothrix thermosphacta	14.1±0.2	7.4±0.9	9.2±0.2	24.0±0.6
Citrobacter freundii	9.1±0.3	9.2±0.2	6.8±0.8	9.7±0.1
Clostridium sporogenes	9.7±0.1	12.9±0.5	20.4±0.4	7.8±0.2
Enterobacter aerogenes	10.0±0.1	12.9±0.9	7.5±0.6	8.9±1.1
Enterococcus faecalis	9.85±0.1	6.4±0.5	7.6±0.2	6.5±0.2
Erwinia carotovora	10.5±0.5	8.1±0.2	8.7±1.2	9.3±0.9
Escherichia coli	13.3±0.2	9.7±0.7	11.0±0.2	12.0±0.8
Flavobacterium suaveolens	11.6±0.6	7.0±0.6	11.0±0.6	10.5±0.2
Klebsiella pneumoniae	10.9±0.3	4.0±0.0	7.8±0.4	10.7±0.5
Lactobacillus plantarum	21.5±0.6	6.2±0.4	12.9±1.7	16.7±2.9
Leuconostoc cremoris	n.d.	n.d.	n.d.	n.d.
Micrococcus luteus	11.7±0.7	6.1±0.1	8.0±0.9	12.8±0.8
Moraxella sp.	10.1±0.6	6.1±0.1	9.0±0.6	6.7±0.2
Proteus vulgaris	8.3±0.3	5.6±0.3	9.8±0.0	8.2±0.1
Pseudomonas aeruginosa	15.5±0.6	5.7±0.3	6.5±0.3	8.4±0.3
Salmonella pullorum	12.9±0.1	6.3±0.2	8.7±0.4	12.0±0.7
Serratia marcescens	22.9±0.8	5.7±0.1	6.8±0.1	12.4±0.9
Staphylococcus aureus	11.5±0.5	5.2±0.1	6.6±0.1	8.2±0.3
Yersinia enterocolitica	11.6±0.4	8.0±0.2	8.2±1.0	11.5±1.1

All values are represented as the mean (mm) ± SEM for duplicate experiments. n.d.: not determined. 4.0: no effect. *: no bacterial growth.

Table 30. Zones of growth inhibition (mm) showing antibacterial activity for a number phytoconstituents from selected plant volatile oils, well diameter of 4.0mm.

Bacterial Strains	Volatile Oil Components			
	α-Humulene	R(+)-Limonene	(±)-Linalool	Menthone
Acinetobacter calcoacetica	4.0±0.0	4.0±0.0	9.3±0.5	9.7±2.3
Aeromones hydrophila	4.0±0.0	4.0±0.0	11.5±0.9	7.0±0.4
Alcaligenes faecalis	4.0±0.0	4.0±0.0	12.1±0.4	6.2±0.5
Bacillus subtilis	4.0±0.0	4.0±0.0	14.0±0.8	7.1±0.3
Beneckea natriegens	4.0±0.0	4.0±0.0	11.4±0.3	5.9±0.4
Brevibacterium linens	4.0±0.0	4.0±0.0	12.5±0.7	4.0±0.0
Brocothrix thermosphacta	4.0±0.0	4.0±0.0	8.1±0.4	6.8±0.4
Citrobacter freundii	4.0±0.0	7.8±0.0	27.5±1.9	7.8±0.6
Clostridium sporogenes	12.0±0.5	10.3±0.0	20.3±0.4	10.7±0.3
Enterobacter aerogenes	4.0±0.0	4.0±0.0	16.7±1.1	4.0±0.0
Enterococcus faecalis	4.0±0.0	7.1±0.2	9.7±0.5	6.3±0.1
Erwinia carotovora	4.0±0.0	7.4±0.1	12.3±0.8	6.5±0.5
Escherichia coli	4.0±0.0	11.2±0.3	13.8±0.3	6.6±0.2
Flavobacterium suaveolens	4.0±0.0	10.6±0.1	15.7±2.4	5.8±0.3
Klebsiella pneumoniae	4.0±0.0	7.0±0.1	12.6±0.3	5.9±0.4
Lactobacillus plantarum	4.0±0.0	4.0±0.0	25.3±0.9	8.8±0.7
Leuconostoc cremoris	n.d.	n.d.	n.d.	n.d.
Micrococcus luteus	4.0±0.0	4.0±0.0	13.4±0.8	7.1±0.3
Moraxella sp.	4.0±0.0	7.9±0.4	10.3±0.9	6.9±0.5
Proteus vulgaris	4.0±0.0	7.4±0.5	12.2±0.9	6.2±0.1
Pseudomonas aeruginosa	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Salmonella pullorum	4.0±0.0	11.2±0.6	7.5±0.5	6.2±0.6
Serratia marcescens	4.0±0.0	6.5±0.1	8.8±0.1	7.1±0.4
Staphylococcus aureus	4.0±0.0	4.0±0.0	9.0±0.4	10.2±1.0
Yersinia enterocolitica	4.0±0.0	7.1±0.2	9.5±0.9	8.0±0.2

All values are represented as the mean (mm) ± SEM for duplicate experiments. n.d.: not determined. 4.0: no effect. *: no bacterial growth.

Table 31. Zones of growth inhibition (mm) showing antibacterial activity for a number phytoconstituents from selected plant volatile oils, well diameter of 4.0mm.

Bacterial Strains	Volatile Oil Components			
	Myrcene	Nerol	β-Ocimene	α-Phellandrene
Acinetobacter calcoacetica	9.6±0.2	11.4±0.5	4.0±0.0	4.0±0.0
Aeromones hydrophila	4.0±0.0	7.7±0.1	4.0±0.0	4.0±0.0
Alcaligenes faecalis	4.0±0.0	7.1±0.4	4.0±0.0	4.0±0.0
Bacillus subtilis	11.8±0.1	12.4±0.2	4.0±0.0	4.0±0.0
Beneckea natriegens	4.0±0.0	11.3±0.5	4.0±0.0	4.0±0.0
Brevibacterium linens	4.0±0.0	11.7±0.6	4.0±0.0	4.0±0.0
Brocothrix thermosphacta	6.5±0.3	9.0±0.9	4.0±0.0	4.0±0.0
Citrobacter freundii	4.0±0.0	7.8±0.4	4.0±0.0	4.0±0.0
Clostridium sporogenes	4.0±0.0	n.d.	8.6±0.9	4.0±0.0
Enterobacter aerogenes	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Enterococcus faecalis	4.0±0.0	7.2±0.5	4.0±0.0	4.0±0.0
Erwinia carotovora	4.0±0.0	7.7±1.2	4.0±0.0	4.0±0.0
Escherichia coli	4.0±0.0	7.6±0.6	4.0±0.0	8.9±0.5
Flavobacterium suaveolens	4.0±0.0	7.0±0.4	4.0±0.0	4.0±0.0
Klebsiella pneumoniae	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Lactobacillus plantarum	4.0±0.0	19.1±0.1	4.0±0.0	4.0±0.0
Leuconostoc cremoris	n.d.	n.d.	n.d.	n.d.
Micrococcus luteus	4.0±0.0	7.4±0.3	4.0±0.0	4.0±0.0
Moraxella sp.	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Proteus vulgaris	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Pseudomonas aeruginosa	4.0±0.0	13.6±1.0	4.0±0.0	4.0±0.0
Salmonella pullorum	8.8±0.6	4.0±0.0	4.0±0.0	4.0±0.0
Serratia marcescens	4.0±0.0	8.5±1.0	4.0±0.0	4.0±0.0
Staphylococcus aureus	4.0±0.0	9.4±0.4	4.0±0.0	8.8±0.1
Yersinia enterocolitica	4.0±0.0	7.1±0.2	4.0±0.0	4.0±0.0

All values are represented as the mean (mm) ± SEM for duplicate experiments. n.d.: not determined. 4.0: no effect. *: no bacterial growth.

Table 32. Zones of growth inhibition (mm) showing antibacterial activity for a number phytoconstituents from selected plant volatile oils, well diameter of 4.0mm.

Bacterial Strains	Volatile Oil Components			
	α-Pinene	β-Pinene	(+)-Sabinene	α-Terpinene
Acinetobacter calcoacetica	4.0±0.0	11.2±0.5	7.8±0.8	4.0±0.0
Aeromones hydrophila	4.0±0.0	7.1±0.3	4.0±0.0	4.0±0.0
Alcaligenes faecalis	4.0±0.0	7.8±0.5	7.7±0.4	4.0±0.0
Bacillus subtilis	4.0±0.0	4.0±0.0	7.4±0.0	4.0±0.0
Beneckea natriegens	4.0±0.0	6.5±0.7	7.7±1.0	6.4±0.1
Brevibacterium linens	4.0±0.0	4.0±0.0	6.5±0.2	6.1±1.1
Brocothrix thermosphacta	4.0±0.0	5.9±0.7	7.6±0.4	4.0±0.0
Citrobacter freundii	6.0±0.3	5.9±0.1	9.5±0.4	4.0±0.0
Clostridium sporogenes	5.7±0.0	7.5±0.2	n.d.	8.8±0.3
Enterobacter aerogenes	9.2±0.1	7.8±0.2	4.0±0.0	4.0±0.0
Enterococcus faecalis	n.d.	n.d.	7.3±0.8	4.0±0.0
Erwinia carotovora	8.7±0.7	4.0±0.0	4.0±0.0	6.3±0.7
Escherichia coli	8.9±0.5	10.8±0.8	4.0±0.0	6.1±0.7
Flavobacterium suaveolens	6.5±0.8	8.4±0.5	4.0±0.0	n.d.
Klebsiella pneumoniae	8.1±0.1	7.9±0.4	4.0±0.0	7.9±0.3
Lactobacillus plantarum	4.0±0.0	4.0±0.0	7.4±0.5	6.4±0.1
Leuconostoc cremoris	n.d.	n.d.	n.d.	n.d.
Micrococcus luteus	7.6±0.8	6.3±0.3	4.0±0.0	4.0±0.0
Moraxella sp.	6.2±0.4	4.8±0.8	4.0±0.0	5.5±0.1
Proteus vulgaris	7.5±0.1	6.6±0.1	4.0±0.0	5.6±0.1
Pseudomonas aeruginosa	4.0±0.0	6.5±0.1	4.0±0.0	6.3±0.4
Salmonella pullorum	7.9±0.5	6.0±0.2	4.0±0.0	16.5±1.1
Serratia marcescens	4.0±0.0	5.4±0.1	4.0±0.0	4.0±0.0
Staphylococcus aureus	8.3±0.0	7.4±0.2	n.d.	4.0±0.0
Yersinia enterocolitica	6.6±0.6	5.8±0.3	4.0±0.0	4.0±0.0

All values are represented as the mean (mm) ± SEM for duplicate experiments. n.d.: not determined. 4.0: no effect. *: no bacterial growth.

Table 33. Zones of growth inhibition (mm) showing antibacterial activity for a number phytoconstituents from selected plant volatile oils, well diameter of 4.0mm.

Bacterial Strains	Volatile Oil Components			
	γ-Terpinene	Terpinen-4-ol	α-Terpineol	Terpinolene
Acinetobacter calcoacetica	4.0±0.0	14.5±0.4	18.7±0.3	10.1±0.2
Aeromones hydrophila	4.0±0.0	24.4±2.1	16.7±0.9	4.0±0.0
Alcaligenes faecalis	4.0±0.0	21.3±1.3	19.3±0.4	4.0±0.0
Bacillus subtilis	6.9±1.1	13.1±0.3	28.8±0.4	12.1±0.1
Beneckea natriegens	4.0±0.0	29.9±0.8	17.6±0.8	4.0±0.0
Brevibacterium linens	4.0±0.0	10.3±0.1	18.4±0.6	4.0±0.0
Brocothrix thermosphacta	4.0±0.0	7.7±0.0	11.3±0.2	7.7±0.6
Citrobacter freundii	4.0±0.0	18.3±0.4	15.4±1.1	4.0±0.0
Clostridium sporogenes	10.1±0.3	9.7±1.65	n.d.	4.0±0.0
Enterobacter aerogenes	6.7±0.5	10.7±0.7	13.4±1.1	4.0±0.0
Enterococcus faecalis	4.0±0.0	17.3±0.4	21.6±1.0	4.0±0.0
Erwinia carotovora	7.7±0.6	14.8±0.7	20.1±0.3	4.0±0.0
Escherichia coli	7.1±0.8	14.4±0.3	16.3±0.3	4.0±0.0
Flavobacterium suaveolens	7.3±0.4	13.4±0.3	21.2±0.2	8.0±0.3
Klebsiella pneumoniae	6.7±1.0	10.7±0.9	19.1±0.1	4.0±0.0
Lactobacillus plantarum	4.0±0.0	14.7±0.5	29.1±0.8	4.0±0.0
Leuconostoc cremoris	n.d.	n.d.	n.d.	n.d.
Micrococcus luteus	4.0±0.0	12.6±0.7	11.2±0.6	9.6±0.8
Moraxella sp.	4.0±0.0	11.4±1.4	18.2±0.2	4.0±0.0
Proteus vulgaris	4.0±0.0	9.7±1.8	20.2±0.2	4.0±0.0
Pseudomonas aeruginosa	4.0±0.0	17.0±1.7	8.9±0.7	4.0±0.0
Salmonella pullorum	4.0±0.0	14.9±0.0	19.2±0.2	4.0±0.0
Serratia marcescens	4.0±0.0	15.2±0.4	11.9±0.3	4.0±0.0
Staphylococcus aureus	10.3±0.3	13.7±0.5	18.2±0.1	4.0±0.0
Yersinia enterocolitica	4.0±0.0	8.6±0.2	20.4±0.4	4.0±0.0

All values are represented as the mean (mm) ± SEM for duplicate experiments. n.d.: not determined. 4.0: no effect. *: no bacterial growth.

Table 34. Zones of growth inhibition (mm) showing antibacterial activity for a number phytoconstituents from selected plant volatile oils, well diameter of 4.0mm.

Bacterial Strains	Volatile Oil Components	
	(-)-Thujone	Thymol
Acinetobacter calcoacetica	8.7±0.5	29.8±1.5
Aeromones hydrophila	12.2±0.1	26.8±1.3
Alcaligenes faecalis	13.5±0.9	32.5±1.2
Bacillus subtilis	12.6±0.3	39.2±2.2
Beneckea natriegens	9.5±0.4	50.1±1.6
Brevibacterium linens	9.8±0.8	42.0±2.0
Brocothrix thermosphacta	12.0±0.3	29.3±1.3
Citrobacter freundii	12.9±0.4	46.5±0.7
Clostridium sporogenes	18.1±2.2	n.d.
Enterobacter aerogenes	13.3±1.0	26.3±3.6
Enterococcus faecalis	10.7±0.2	30.5±0.4
Erwinia carotovora	11.0±1.2	32.0±1.0
Escherichia coli	12.2±0.5	34.3±5.4
Flavobacterium suaveolens	11.8±0.4	25.8±0.8
Klebsiella pneumoniae	11.1±0.1	40.0±3.4
Lactobacillus plantarum	13.4±1.2	*
Leuconostoc cremoris	n.d.	n.d.
Micrococcus luteus	11.3±0.7	53.1±1.3
Moraxella sp.	10.0±1.8	39.1±0.9
Proteus vulgaris	12.6±0.2	33.5±6.2
Pseudomonas aeruginosa	9.3±0.7	13.4±1.4
Salmonella pullorum	11.5±0.2	31.5±0.3
Serratia marcescens	7.5±0.4	42.6±0.5
Staphylococcus aureus	9.4±0.1	31.6±0.4
Yersinia enterocolitica	9.0±0.3	27.7±0.1

All values are represented as the mean (mm) ± SEM for duplicate experiments. n.d.: not determined. 4.0: no effect. *: no bacterial growth.

Citronellal

Citronellal, a constituent of the volatile oils of geranium [32.92%] (Table 12) and melissa [24.92%] (Table 16) demonstrated limited inhibitory activity against the test a limited number of organisms (Table 28). The degree of sensitivity being *B. subtilis* > *C. sporogenes* > *F. suaveolens* > *M. luteus* > *P. aeruginosa* > *E. faecalis*. The antimicrobial activity of the volatile oil of *Eucalyptus globulus* Labill. (Myrtaceae) has been ascribed to its monoterpenes, principally to citronellol and citronellal (Low *et al.,* 1974). Citronellal was more effective against the microorganism *B. subtilis* than the antibiotics used in this study, except for the antibiotic neomycin, Table 23.

p-Cymene

p-Cymene, a constituent of the volatile oils of black pepper [1.63%] (Table 10), geranium [0.15%] (Table 12), lovage leaf (6 months) [0.10%] (Table 14), lovage stem [0.61%] (Table 15), monarda [10.63%] (Table 17), nutmeg [1.87%] (Table 18), nutmeg [*Serva*] [3.94%] (Table 19), oregano [12.7%] (Table 20) and thyme [25.51%] (Table 21) did not demonstrate an ability to inhibit the growth of any of the test organisms (Table 28).

Eugenol

Eugenol, a constituent of the volatile oils of black pepper [5.56%] (Table 10) and clove [86.48%] (Table 11) inhibited the growth of all the tested organisms to some degree, as shown in Table 29. The order of sensitivities of the bacteria were found to be *S. marcescens* > *B. subtilis* > *L. plantarum* > *B. natriegens* > *A. hydrophila* > *P. aeruginosa* > *A. calcoacetica* > *B. thermosphacta* > *E. coli* > *S. pullorum* > *B. linens* > *A. faecalis* > *M. luteus* > *F. suaveolens* > *Y. enterocolitica* > *S. aureus* > *K. pneumoniae* > *E. carotovora* > *Moraxella sp.* > *E. aerogenes* > *E. faecalis* > *C. sporogenes* > *C. freundii* > *P. vulgaris*. Eugenol is a phenol compound and like other phenols it may be considered to be a membrane-active antibacterial substance (Hamilton, 1971).

Eugenol was more effective than the antibiotics used in this study against the microorganisms *B. subtilis*, *B. natriegens*, *S. marcescens*, *A. calcoacetica* except for the antibiotics erythromycin, streptomycin and neomycin; *A. hydrophila* except for

the antibiotics chloramphenicol and tetracycline and *A. faecalis* except for the antibiotic neomycin, Table 23.

Geraniol

Geraniol, a constituent of the volatile oils of black pepper [0.54%] (Table 10), geranium [12.01%] (Table 12), nutmeg [0.13%] (Table 18) and nutmeg [*Serva*] [0.7%] (Table 19) demonstrated antibacterial activity against all the tested organisms except for *K. pneumoniae*, Table 29.

Geraniol was more effective than the antibiotics used in this study, in the case of the microorganisms *A. calcoacetica* except for the antibiotics erythromycin, streptomycin, neomycin and ampicillin; *A. hydrophila* except for the antibiotics chloramphenicol, tetracycline and neomycin; *A. faecalis* except for the antibiotic neomycin; *B. subtilis* and *B. natriegens* except for the antibiotics neomycin and ampicillin; *E. aerogenes* except for the antibiotics chloramphenicol and tetracycline; *E. coli* except for the antibiotic novobiocin and *Moraxella sp.* except for the antibiotics chloramphenicol, streptomycin, neomycin and ampicillin, Table 23..

Geranyl Acetate

Geranyl acetate, a constituent of the volatile oil of geranium [0.44%] (Table 12) demonstrated antimicrobial activity against all the tested microorganisms, Table 29. The order of sensitivity was found to be *C. sporogenes* > *L. plantarum* > *B. linens* > *E. coli* ~ *F. suaveolens* > *B. subtilis* ~ *B. natriegens* > *A. faecalis* > *A. calcoacetica* > *P. vulgaris* > *B. thermosphacta* > *A. hydrophila* ~ *Moraxella sp.*> *E. carotovora* ~ *S. pullorum* > *Y. enterocolitica* > *M. luteus* > *K. pneumoniae* > *E. faecalis* > *E. aerogenes* > *C. freundii* ~ *S. marcescens* > *S. aureus* > *P. aeruginosa.*

Geranyl acetate was more effective than the antibiotics used in this study, in the case of the microorganisms *B. natriegens, A. calcoacetica* except for the antibiotics erythromycin, streptomycin and neomycin; *A. hydrophila* except for the antibiotics chloramphenicol, tetracycline and neomycin; *A. faecalis* and *B. subtilis* except for the antibiotic neomycin; *E. coli* except for the antibiotic novobiocin; *F. suaveolens* except for the antibiotics novobiocin, oleandomycin and streptomycin; L. plantarum except for the antibiotics erythromycin, oleandomycin and tetracycline; *Moraxella sp.* except for the antibiotics chloramphenicol, streptomycin, neomycin and ampicillin; *P. vulgaris* except for the antibiotics streptomycin, tetracycline and

neomycin and *S. pullorum* except for the antibiotics chloramphenicol, streptomycin and neomycin, Table 23.

cis-3-Hexan-1-ol

cis-Hex-3-an-1-ol caused mild to moderate inhibitory activity against the microorganisms under test. *Brocothrix thermosphacta* was the most sensitive strain, Table 29.

cis-Hex-3-an-1-ol was more effective than the antibiotics used in this study, in the case of the microorganisms *A. hydrophila* except for the antibiotics chloramphenicol, tetracycline and neomycin; *A. faecalis* except for the antibiotic neomycin, *B. subtilis* and *B. natriegens* except for the antibiotics neomycin and ampicillin; *B. thermaphacta*, *E. coli* except for the antibiotic novobiocin and *L. plantarum* except for the antibiotics oleandomycin and tetracycline, Table 23.

α-Humulene

α-Humulene, a constituent of the volatile oils of black pepper [2.16%] (Table 10), clove [0.66%] (Table 11), geranium [0.25%] (Table 12) and nutmeg [*Serva*] [0.61%] (Table 19) was only effective at inhibiting the growth of the food poisoning organism *C. sporogenes*, Table 30. It was ineffective against *A. calcoacetica*, *A. hydrophila*, *A. faecalis*, *B. subtilis*, *B. natriegens*, *B. linens*, *B. thermosphacta*, *C. freundii*, *E. aerogenes* and *S. marcescens*.

R (+)-Limonene

Limonene, a constituent of the volatile oils of geranium [0.21%] (Table 12), melissa [57.53%] (Table 16), monarda [0.38%] (Table 17), nutmeg [3.87%] (Table 18), nutmeg [*Serva*] [10.00%] (Table 19), oregano [0.77%] (Table 20) and thyme [1.14%] (Table 21) demonstrated activity against a number of test organisms, Table 30. The sensitivity being *E. coli* ~ *S. pullorum* > *F. suaveolens* > *C. sporogenes* > *Moraxella sp.* > *C. freundii* > *E. carotovora* > *P. vulgaris* > *E. faecalis* > *Y. enterocolitica* > *K. pneumoniae* > *S. marcescens*. It was unable to inhibit the growth of *A. calcoacetica*, *A. hydrophila*, *A. faecalis*, B. *subtilis*, *B. natriegens*, *B. linens*, *B. thermosphacta*, *E. aerogenes*, *L. plantarum*, *M. luteus*, *P. aeruginosa* and *S. aureus*.

Limonene was more effective than the antibiotics used in this study against the microorganisms *E. coli* except for the antibiotic novobiocin; *F. suaveolens* except

for the antibiotics novobiocin, Oleandomycin and streptomycin; *P. vulgaris* except for the antibiotics streptomycin, tetracycline and neomycin and *S. pullorum* except for the antibiotics chloramphenicol, streptomycin and neomycin, Table 23.

(±)-Linalool

Linalool, a constituent of the volatile oils of geranium [6.62%] (Table 12), melissa [0.60%] (Table 16), monarda [0.30%] (Table 17), nutmeg [0.47%] (Table 18), nutmeg [*Serva*] [1.20%] (Table 19), oregano [0.60%] (Table 20) and thyme [4.56%] (Table 21) was found to be effective against all the test organisms except *P. aeruginosa*, Table 30. The order of sensitivity was found to be *L cremoris* > *C. freundii* > *L. plantarum* > *C. sporogenes* > *E. aerogenes* > *F. suaveolens* > *B. subtilis* > *E. coli* > *M. luteus* > *K. pneumoniae* > *B. linens* > *E. carotovora* > *P. vulgaris* > *A. faecalis* > *A. hydrophila* > *B. natriegens* > *Moraxella sp.* > *E. faecalis* > *Y. enterocolitica* > *A. calcoacetica* > *S. aureus* > *S. marcescens* > *B. thermosphacta* > *S. pullorum*.

Linalool was more effective than the antibiotics used in this study against the microorganisms *A. calcoacetica* except for the antibiotics erythromycin, streptomycin and neomycin; *A. hydrophila* except for the antibiotics chloramphenicol, tetracycline, neomycin; *A. faecalis* except for the antibiotic neomycin; *B. subtilis*, *B. natriegens*, *C. freundii*, *E. aerogenes*, *E. coli* and *F. suaveolens* except for the antibiotic novobiocin; *L. plantarum* and *P. vulgaris* except for the antibiotics streptomycin and neomycin, Table 23.

Menthone

Menthone, a constituent of the volatile oil of geranium [1.75%] (Table 12) was shown to be effective against the majority of organisms, Table 30. The degree of sensitivities being *C. sporogenes* > *S. aureus* > *A. calcoacetica* > *L. plantarum* > *Y. enterocolitica* > *C. freundii* > *B. subtilis* ~ *M. luteus* ~ *S. marcescens* > *A. hydrophila* > *Moraxella sp.* > *B. thermosphacta* > *E. coli* > *E. carotovora* > *E. faecalis* > *A. faecalis* ~ *P. vulgaris* ~ *S. pullorum* > *B. natriegens* ~ *K. pneumoniae* ~ *F. suaveolens*.

Menthone was more effective than the antibiotics used in this study in the case of the microorganisms *A. calcoacetica* except for the antibiotics erythromycin, streptomycin and neomycin; *A. hydrophila* except for the antibiotics

chloramphenicol, tetracycline and neomycin; *A. faecalis* except for the antibiotic neomycin; *B. subtilis* and *B. natriegens* except for the antibiotics neomycin and ampicillin and *E. coli* except for the antibiotic novobiocin, Table 23.

Myrcene

Myrcene, a constituent of the volatile oils of geranium [0.17%] (Table 12), lovage leaf (3 months) [2.32%] (Table 13), lovage leaf (6 months) [2.98%] (Table 14), lovage stem [2.04%] (Table 15), melissa [0.62%] (Table 16), monarda [0.19%] (Table 17), nutmeg [1.89%] (Table 18), nutmeg [*Serva*] [1.24%] (Table 19), oregano [1.45%] (Table 20) and thyme [1.22%] (Table 21) was only active against *A. calcoacetica*, *B. subtilis*, *B. thermosphacta* and *S. marcescens*, Table 31. The low activity of this component has been reported previously (Onawunmi *et al.*, 1984). However, when mixed with either geranial (α-citral) or neral (β-citral), myrcene was reported as enhancing the activities of these components, therefore acting synergistically (Onawunmi *et al.*, 1984).

Myrcene exhibited limited activity in comparison to the antibiotics used in this study, being only more effective against the microorganisms *A. calcoacetica* except for the antibiotics erythromycin, streptomycin and neomycin and *B. subtilis* except for the antibiotic neomycin, Table 23.

Nerol

Nerol, a constituent of the volatile oil of melissa [4.34%] (Table 16) was effective against 18/23 organisms tested, Table 31. The most resistant bacteria were found to be *E. aerogenes*, *K. pneumoniae*, *Moraxella sp.*, *P. vulgaris* and *S. pullorum*.

Nerol was more effective than the antibiotics used in this study against the microorganisms *A. calcoacetica* except for the antibiotics erythromycin, streptomycin and neomycin; *A. faecalis* and *B. subtilis* except for the antibiotic neomycin; *B. natriegens*; *E. coli* except for the antibiotic novobiocin; *P. aeruginosa* except for the antibiotic neomycin and ampicillin and *L. plantarum*, Table 23.

β-Ocimene

β-Ocimene, a constituent of the volatile oils of geranium [0.13%] (Table 13) was found to be ineffective inhibiting the growth of the bacterial strains except for *Clostridium sporogenes* which was only moderately inhibited, Table 31.

α-Phellandrene

α-Phellandrene, a constituent of the volatile oils of black pepper [2.74%] (Table 10), lovage leaf (3 months) [0.64%] (Table 13), lovage leaf (6 months) [0.83%] (Table 14), lovage stem [0.28%] (Table 15), nutmeg [0.67%] (Table 18) and nutmeg [*Serva*] [0.51%] (Table 19) was found to be effective against only two test organisms, significantly the animal pathogen and food poisoning organisms *E. coli* and *S. aureus*, Table 31.

α-Pinene

α-Pinene, a constituent of the volatile oils of black pepper [6.33%] (Table 10), geranium [0.54%] (Table 12), lovage leaf (3 months)[0.75%] (Table 13), lovage leaf (6 months) [0.85%] (Table 14), lovage stem [0.21%] (Table 15), melissa [0.35%] (Table 16), monarda [0.28%] (Table 17), nutmeg [22.04%] (Table 18), nutmeg [*Serva*] [11.39%] (Table 19) and thyme [2.12%] (Table 21) was found to be effective against a number of microorganisms, Table 32. The order of sensitivity of the microorganisms were *E. aerogenes* > *E. coli* > *E. carotovora* > *S. aureus* > *K. pneumoniae* > *S. pullorum* > *M. luteus* > *P. vulgaris* > *Y. enterocolitica* > *F. suaveolens* > *Moraxella sp.* > *C. freundii* > *C. sporogenes.*

α-Pinene was more effective against the microorganisms *E. coli* except for the antibiotic novobiocin and *P. vulgaris* except for the antibiotics streptomycin, tetracycline and neomycin, Table 23.

β-Pinene

β-Pinene, a constituent of the volatile oils of lovage leaf (3 months) [0.19%] (Table 13), lovage leaf (6 months) [0.19%] (Table 14), lovage stem [1.76%] (Table 15), nutmeg [21.53%] (Table 18), nutmeg [*Serva*] [9.39%] (Table 19), thyme [0.10%] (Table 21) demonstrated antibacterial activity, Table 32. The degree of sensitivity being *A. calcoacetica* > *E. coli* > *F. suaveolens* > *K. pneumoniae* > *A. faecalis* ~ *E. aerogenes* > *C. sporogenes* > *S. aureus* > *A. hydrophila* > *P. vulgaris* >

B. natriegens > *P. aeruginosa* > *M. luteus* > *S. pullorum* > *B. thermosphacta* > *C. freundii* > *Y. enterocolitica* > *S. marcescens* > *Moraxella sp.*.

β-Pinene was more effective against the microorganisms *A. hydrophila* except for the antibiotics chloramphenicol, tetracycline and neomycin; *A. faecalis* except for the antibiotic neomycin; *B. natriegens* except for the antibiotics neomycin and ampicillin; *E. coli* except for the antibiotic novobiocin and *P. vulgaris* except for the antibiotics streptomycin, tetracycline and neomycin than the antibiotics used in this study, Table 23.

(+)-*Sabinene*

Sabinene, a constituent of the volatile oils of black pepper [0.15%] (Table 10), lovage leaf (3 month)[0.22%] (Table 13), lovage leaf (6 months) [0.22%] (Table 14), melissa [0.52%] (Table 16), nutmeg [15.39%] (Table 18) and nutmeg [*Serva*] [13.59%] (Table 19) demonstrated antibacterial activity against the microorganisms *C. freundii*, *A. calcoacetica*, *A. faecalis*, *B. natriegens*, *B. thermosphacta*, *B. subtilis*, *L. plantarum*, *E. faecalis* and *B. linens*. *C. sporogenes*, Table 32.

Sabinene was more effective than the antibiotics used in this study against the microorganisms *A. faecalis* except for the antibiotics neomycin; *B. subtilis* and *B. natriegens* except for the antibiotics neomycin and ampicillin, Table 25.

α-Terpinene

α-Terpinene, a constituent of the volatile oils of monarda [0.88%] (Table 17), nutmeg [1.24%] (Table 18), nutmeg [*Serva*] [1.71%] (Table 19), oregano [1.01%] (Table 20) and thyme [0.44%] (Table 21) effected the growth of the test organisms *S. pullorum* > *C. sporogenes* > *K. pneumoniae* > *B. natriegens* > *L. plantarum* > *E. carotovora* > *P. aeruginosa* > *B. linens* > *E. coli* > *P. vulgaris* > *Moraxella sp.*, Table 32. *A. calcoacetica*, *A. hydrophila*, *A. faecalis*, *B. subtilis*, *B. thermosphacta*, *C. freundii*, *E. aerogenes*, *M. luteus*, *S. marcescens*, *S. aureus*, *E. faecalis* and *Y. enterocolitica* were unaffected, growing to confluence.

α-Terpinene was more effective than the antibiotics used in this study against the microorganisms *B. natriegens* except for the antibiotics neomycin and ampicillin; *E. coli* except for the antibiotic novobiocin; *P. vulgaris* except for the antibiotic streptomycin, tetracycline and neomycin and *S. pullorum* except for the antibiotics streptomycin and neomycin, Table 23.

γ-Terpinene

γ-Terpinene, a constituent of the volatile oils of lovage leaf (3 months) [0.14%] (Table 13), lovage leaf (6 months)[0.24%] (Table 14), lovage stem [0.59%] (Table 15), monarda [0.94%] (Table 17), nutmeg [1.80%] (Table 18), nutmeg [*Serva*] [2.19%] (Table 19), oregano [2.09%] (Table 20) and thyme [4.44%] (Table 21) demonstrated some degree of activity against *S. aureus* > *C. sporogenes* > *E. carotovora* > *F. suaveolens* > *E. coli* > *B. subtilis* > *E. aerogenes* > *K. pneumoniae*, Table 33. All the remaining organisms, except for *L. cremoris* which was not tested, were unaffected by the component.

γ-Terpinene was more effective against the microorganism *E. coli* than the antibiotics used in this study except for the antibiotic novobiocin, Table 23.

Terpinen-4-ol

Terpinen-4-ol, a constituent of the volatile oils of black pepper [0.24%] (Table 10), lovage leaf (3 months) [0.16%] (Table 13), lovage leaf (6 months) [0.37%] (Table 14), lovage stem [0.81%] (Table 15), monarda [1.23%] (Table 17), nutmeg [5.75%] (Table 18), nutmeg [*Serva*] [4.85%] (Table 19), oregano [0.82%] (Table 20) and thyme [0.23%] (Table 21) was effective against all the test organisms, Table 33. The order of sensitivity being *B. natriegens* > *A. hydrophila* > *A. faecalis* > *C. freundii* > *E. faecalis* > *P. aeruginosa* > *S. marcescens* > *S. pullorum* > *E. carotovora* > *L. plantarum* > *A. calcoacetica* > *E. coli* > *S. aureus* > *F. suaveolens* > *B. subtilis* > *M. luteus* > *Moraxella sp.* > *K. pneumoniae* > *E. aerogenes* > *B. linens* > *P. vulgaris* > *C. sporogenes* > *Y. enterocolitica* > *B. thermosphacta.*

Terpinen-4-ol was more effective than the antibiotics used in this study against the microorganisms *A. calcoacetica* except for the antibiotics erythromycin, streptomycin and neomycin; *A. hydrophila*, *A. faecalis*; *B. subtilis* except for neomycin; *B. natriegens*, *C. freundii*; *E. faecalis* except for the antibiotic ampicillin; *E. carotovora* except for the antibiotics streptomycin, neomycin and ampicillin; *E. coli* except for novobiocin; *F. suaveolens* except for the antibiotics novobiocin and streptomycin; *L. plantarum* except for the antibiotics erythromycin, oleandomycin and tetracycline; *Moraxella sp.* except for the antibiotics chloramphenicol and streptomycin; *P. vulgaris* except for the antibiotics streptomycin, tetracycline and

neomycin; *S. pullorum* except for the antibiotics streptomycin and neomycin and *P. aeruginosa*, Table 23.

α-Terpineol

α-Terpineol, a constituent of the volatile oils of black pepper [4.74%] (Table 10), geranium [0.57%] (Table 12), lovage leaf (3 months)[1.49%] (Table 13), lovage leaf (6 months) [1.27%] (Table 14), lovage stem [1.21%] (Table 15), monarda [0.14%] (Table 17), nutmeg [0.73%] (Table 18), nutmeg [*Serva*] [6.78%] (Table 19), oregano [0.75%] (Table 20) and thyme [0.4%] (Table 21) was effective against all the microorganisms, Table 33. The order of sensitivity being *L. plantarum* > *B. subtilis* > *E. faecalis* > *F. suaveolens* > *Y. enterocolitica* > *P. vulgaris* > *E. carotovora* > *A. faecalis* > *S. pullorum* > *K. pneumoniae* > *A. calcoacetica* > *B. linens* > *Moraxella sp.* > *S. aureus* > *B. natriegens* > *A. hydrophila* > *E. coli* > *C. freundii* > *E. aerogenes* > *S. marcescens* > *B. thermosphacta* > *M. luteus* > *P. aeruginosa.*

α-Terpineol was more effective than the antibiotics used in this study against the microorganisms *A. calcoacetica* except for the antibiotic streptomycin; *A. hydrophila* except for the antibiotics chloramphenicol and tetracycline; *A. faecalis*, *B. subtilis*, *B. natriegens*; *C. freundii* except for the antibiotic ampicillin; *E. aerogenes* except for the antibiotics chloramphenicol and tetracycline; *E. faecalis* except for the antibiotic ampicillin; *E. carotovora*, *E. coli*; *F. suaveolens* except for the antibiotic novobiocin; *K. pneumoniae* except for the antibiotic erythromycin; *L. plantarum*, *Moraxella sp.*, *P. vulgaris*; *S. pullorum* except for the antibiotic streptomycin; *S. aureus* except for the antibiotics tetracycline and ampicillin and *Y. enterocolitica*, Table 23.

Terpinolene

Terpinolene, a constituent of the volatile oils of lovage leaf (3 months) [0.38%] (Table 13), lovage leaf (6 month) [0.36%] (Table 14), lovage stem [0.37%] (Table 15), nutmeg [0.93%] (Table 18), nutmeg [*Serva*] [1.90%] (Table 19), oregano [0.27%] (Table 20) and thyme [0.49%] (Table 21) was active against 5 organisms: *A. calcoacetica*, *B. subtilis*, *B. thermosphacta*, *F. suaveolens* and M. *luteus*, Table 33.

Terpinolene was more effective than the antibiotics used in this study against the microorganisms *A. calcoacetica* except for the antibiotics erythromycin,

streptomycin and neomycin and *B. subtilis* except for the antibiotic neomycin, Table 23.

(-)-Thujone

Thujone was more effective than the antibiotics used in this study against the microorganisms *A. calcoacetica* except for the antibiotics erythromycin, streptomycin and neomycin; *A. hydrophila* except for the antibiotics chloramphenicol, tetracycline and neomycin; *A. faecalis* and *B. subtilis* except for the antibiotics neomycin; *B. natriegens* except for the antibiotics neomycin and ampicillin; *C. freundii* except for the antibiotics streptomycin, tetracycline and ampicillin; *E. aerogenes* except for the antibiotics chloramphenicol and tetracycline; *E. coli* except for the antibiotic novobiocin; *F. suaveolens* except for the antibiotics novobiocin, oleandomycin and tetracycline; *P. vulgaris* except for the antibiotics streptomycin and neomycin and *S. pullorum* except for the antibiotics chloramphenicol, streptomycin and neomycin, Table 23.

Thymol

Thymol, a constituent of the volatile oils of monarda [70.59%] (Table 17), oregano [1.36%] (Table 20) and thyme [48.0%] (Table 21) demonstrated activity against all the test organisms tested at 1g mL^{-1}, Table 34. *L. plantarum* and *L. cremoris* were the most sensitive, being completely inhibited from growing in the media, while *P. aeruginosa* appeared to be the most resistant.

Thymol was more effective than the antibiotics used in this study against all the microorganisms used with only a limited number of exceptions. These exceptions included the antibiotic neomycin against *B. linens*, ampicillin against *E. faecalis*, novobiocin against *F. suaveolens* and the antibiotics neomycin and ampicillin against *P. aeruginosa*, Table 23.

3.4. Discussion

3.4.1. Antibacterial Activity of Plant Volatile Oils

All the bacterial strains demonstrated some degree of sensitivity to the plant volatile oils tested. In the case of a number of microorganisms, which appeared unaffected by one or more oils, the stronger acting oils, e.g. thyme volatile oil,

inhibited them. The activities of the oils were in a number of cases superior and wider ranging than for the antibiotics, as shown in Table 23.

The data presented in this study are in general agreement with previously reported studies in the case of black pepper (Deans and Ritchie, 1987; Ouattara *et al.,* 1997), clove (Briozzo *et al.,* 1989; Cai and Wu, 1996; Hao *et al.,* 1998; Smith-Palmer *et al.,* 1998), geranium (Pattnaik *et al.,* 1996), melissa (Larrondo *et al.,* 1995), nutmeg, oregano (Kivanc and Akgül, 1986) and thyme (Kivanc and Akgül, 1986; Smith-Palmer *et al.,* 1998).

From the number of oils tested, it was not possible to conclusively determine whether the hypothesis Gram -ve bacteria are more resistant than Gram -ve bacteria as proposed by Zaika (1988) or the hypothesis that the susceptibility by bacteria to plant volatile oils and the Gram reaction appears to have little influence on growth inhibition as proposed by Deans (Deans and Ritchie, 1987; Deans *et al.,* 1995). The oils of black pepper, clove and oregano did not appear to be more effective against either Gram +ve nor Gram -ve microorganisms, contrary to the data presented by Hussein (1990), Smith-Palmer *et al.* (1998) and Zaika (1988). However, in the case of geranium, melissa and thyme volatile oils, there did appear to be a preference in Gram reaction susceptibility. The Gram -ve organisms were the most sensitive to the actions of these oils. This suggests that differences in the biochemistry of the bacterial cell wall/membrane contribute in some fashion to the susceptibility of such microorganisms to these particular oils.

3.4.2. Nature of Antibacterial Activity

A correlation of the antimicrobial activity of the checked compounds with their chemical structure, functional groups and configuration, suggest a number of observations.

3.4.2.1. Phenolic compounds (e.g. carvacrol and thyme) were highly active

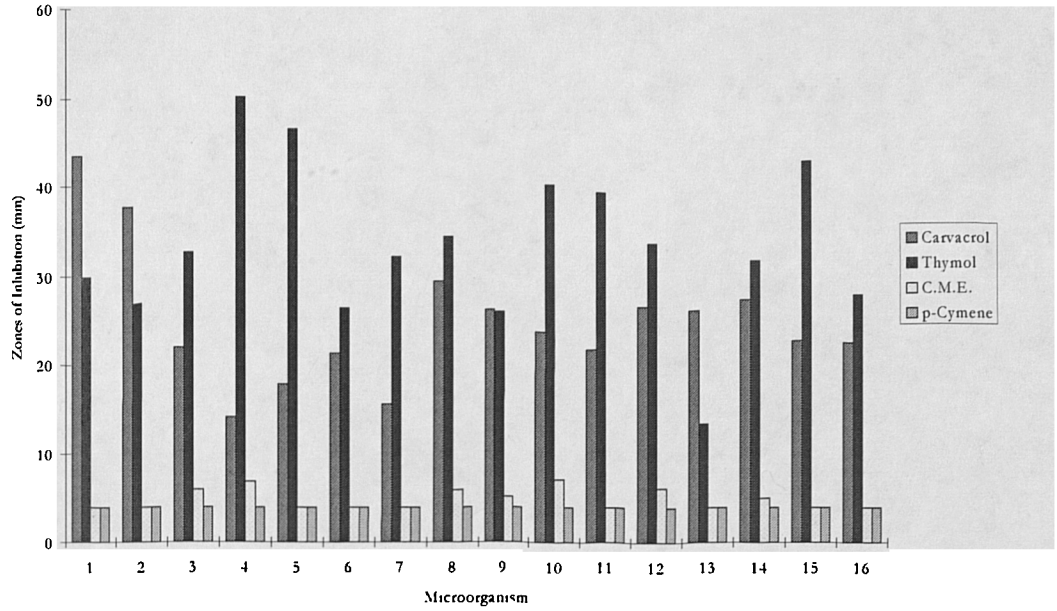
Members of this class known to be either bactericidal or bacteriostatic agents depending upon the concentration used (Pelczar *et al.,* 1988). At high concentrations phenol exhibits rapid cytocidal effects by penetrating and disrupting the cell wall and acting as a gross cytoplasmic poison (Prindle, 1983), while at lower concentrations

cell death occurs. The action of phenols is thought to be multifaceted, involving physically damaging the permeability barrier in the bacterial cell wall (Judis, 1963) resulting the leakage of volatile components such as amino acids (Gale and Taylor, 1947; Haydon, 1956; Maurice, 1952) and the activation (Pulvertaft and Lumb, 1948) and inactivation of enzymes (Bach and Lambert, 1937a; Bach and Lambert, 1937b; Roberts and Rahn, 1946; Sykes, 1939).

The phenolics thymol (**48**), carvacrol (**10**) and eugenol (**19**) were strongly active despite their relatively low capacity to dissolve in water, in agreement with published data (Belaiche *et al.,* 1995; Charai *et al.,* 1996; Hili *et al.,* 1997; Jeongmok *et al.,* 1995; Knobloch *et al.,* 1988; Lattaoui and Tantaoui-Elaraki, 1994; Lis-Balchin and Deans, 1997; Mahmoud, 1994; Meena and Sethi, 1994; Nadal *et al.,* 1973; Pauli and Knobloch, 1987; Shapiro *et al.,* 1994; Sivropoulou *et al.,* 1996; Suresh *et al.,* 1992).

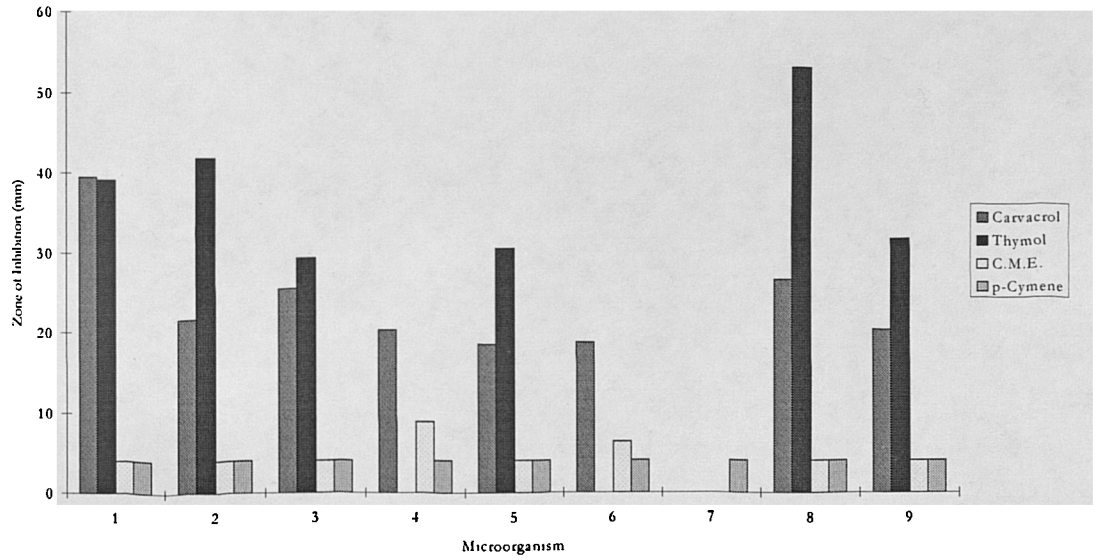
The importance of the hydroxyl group in the phenolic structure was confirmed in terms of activity when carvacrol (**10**) was compared to its methyl ether (**11**). Furthermore, the relative position of the hydroxyl group exerted an influence upon the components effectiveness as seen in the difference in activity between carvacrol and thymol against Gram negative and Gram positive microorganisms, Figure 21 and Figure 22 respectively. Additionally, the importance of the phenolic ring was demonstrated by the lack of activity of the monoterpene cyclic hydrocarbon *p*-cymene (**18**), also seen in Figure 21 and Figure 22.

The high activity of the phenolic components tested may additionally explained in terms of the alkyl substitution into the phenol nucleus, which is known to enhance the antimicrobial activity of phenols (Pelczar *et al.,* 1988; Prindle *et al.,* 1968). The introduction of alkylation has been proposed to alter the distribution ratio between the aqueous and the non-aqueous phases - including bacterial phases - by reducing the surface tension or altering the species selectivity (Read and Mullin, 1928; Read and Miller, 1932; Schaffer and Tilley, 1926; Schaffer and Tilley, 1927; Woodward *et al,* 1933). Data suggests alkyl substituted phenolic compounds form phenoxyl radicals which interact with isomeric alkyl substituents (Pauli and Knoblock, 1987). This does not occur with etherified/esterified isomeric molecules, explaining their relative



Key: 1: *A. calcoacetica*; 2: *A. hydrophila*; 3: *A. faecalis*; 4: *B. natriegens*; 5: *C. freundii*; 6: *E. aerogenes*; 7: *E. carotovora*; 8: *E. coli*; 9: *F. suaveolens*; 10: *K. pneumoniae*; 11: *Moraxella sp.*; 12: *P. vulgaris*; 13: *P. aeruginosa*; 14: *S. pullorum*; 15: *S. marcescens*; 16: *Y. enterocolitica*. C.M.E.: Carvacrol methyl ether.

Figure 21. The antimicrobial efficacy of carvacrol, carvacrol methyl ether, *p*-cymene and thymol against Gram -ve organisms.



Key: 1: *B. subtilis*; 2: *B. linens*; 3: *B. thermosphacta*; 4: *C. sporogenes*; 5: *E. faecalis*; 6:*L. plantarum*; 7: *L. cremoris*; 8: *M. luteus*; 9: *S. aureus*. C.M.E.: Carvacrol methyl ether.

Figure 22. The antimicrobial efficacy of carvacrol, carvacrol methyl ether, *p*-cymene and thymol against Gram +ve organisms.

3.4.2.2. Introduction of an acetate function increased activity

The presence of an acetate moiety in the structure appeared to increase the activity of the parent compound. In the case of geraniol, the geranyl acetate demonstrated an increase in activity against the test microorganisms, as shown in Table 29. Only *C. sporogenes* was found to be more resistant to the acetate. A similar tendency was identified in the case of borneol and bornyl acetate, Table 26. Borneol was less active than the acetate except against *A. hydrophila*, *B. subtilis*, *B. natriegens*, *E. coli*, *F. suaveolens* and *S. marcescens* but only the acetate was capable of affecting the growth of the bacterium *M. luteus*.

3.4.2.3. Alcohol functions demonstrated mild activity

Alcohols are known to possess bactericidal rather than bacteriostatic activity against vegetative cells. The alcohol terpenoids in this study did exhibit activity against the test microorganisms, potentially acting as either protein denaturing agents (Pelczar *et al.,* 1988), solvents or dehydrating agents.

3.4.2.4. Aldehydes demonstrated mild activity

Aldehydes, notably formaldehyde and glutaraldehyde, are known to possess powerful antimicrobial activity. It has been proposed that an aldehyde group conjugated to a carbon to carbon double bond is a highly electro-negative arrangement which may explain their activity (Moleyar and Narasimham, 1986), suggesting an increase in electro-negativity increases the antibacterial activity (Kurita *et al.,* 1979; Kurita *et al.,* 1981). Such electro-negative compounds may interfere in biological processes involving electron transfer and react with vital nitrogen components, e.g. proteins and nucleic acids and therefore inhibit the growth of the microorganisms. The aldehydes citral (*cis+trans*) displayed moderate activity against the test microorganisms while citronellal was only active against *B. subtilis*, *C. sporogenes, F. suaveolens, M. luteus* and *P. aeruginosa* (Table 28).

3.4.2.5. Ketones demonstrated moderate activity

A number of the components tested are ketones. The presence of an oxygen function in the framework increases the antimicrobial properties of terpenoids (Naigre *et al.,* 1996). From this study by using the contact method, the bacteriostatic

and fungistatic action of terpenoids was increased when carbonylated. Menthone was shown to have modest activity, *C. sporogenes* and *S. aureus* being the most significantly affected (Table 30).

3.4.2.6. Alkyl Substitution into non-phenolic structure increased activity

An increase in activity dependent upon the type of alkyl substituent incorporated into a non-phenolic ring structure appeared to occur in this study. An alkenyl substituent [1-methylethenyl] resulted in increased antibacterial activity, as seen in limonene [1-methyl-4-(1-methylethenyl)-cyclohexene], as compared to an alkyl [1-methylethyl] substituent as in *p*-cymene [1-methyl-4-(1-methylethyl)-benzene]. As shown in Table 28 and Table 30, the inclusion of a double bond increased the activity of limonene relative to *p*-cymene, which demonstrated no activity against the test bacteria. In addition, the susceptible organisms were principally Gram -ve, which suggests alkylation influences Gram reaction sensitivity of the bacteria.

The importance of the antimicrobial activity of alkylated phenols in relation to phenol has been reported (Pauli and Kubeczka, personal communication). Their data suggests that an allylic side chain seems to enhance a component's inhibitory effects and chiefly against Gram -ve organisms.

3.4.2.7. Miscellaneous Factors

There were insufficient data to confirm published observations proposed by Hinou *et al.* (1989). These included whether α-isomers are inactive relative to β-isomers, e.g. α-pinene; *cis*-isomers are inactive contrary to *trans*-isomers, e.g. geraniol and nerol; compounds with methyl-isopropyl cyclohexane rings are the most active or unsaturation of the cyclohexane ring further increases the antibacterial activity, e.g. terpinolene, terpineol and terpineolene. Nor was there enough information to confirm α- or *cis*-isomers and non-typical terpene C_{10} structures generate negative results, e.g. citronellol or nerolidol.

3.5. Conclusions

The mechanisms underpinning the antibacterial activity are difficult to determine from this data. Investigations into the effects of terpenoids upon isolated

bacterial membranes (Knobloch *et al.,* 1986) suggests that their activity is a function of the constituent terpenes lipophilic properties, the potency of their functional groups (Knobloch *et al.,* 1988) and their aqueous solubility (Knobloch *et al.,* 1988). Their site of action appears to be at the phospholipid bilayer, caused by biochemical mechanisms catalysed by the phospholipid bilayers of the cell. These processes include the inhibition of electron transport, protein translocation, phosphorylation steps and other enzyme-dependent reactions (Knobloch *et al.,* 1986). Their activity in whole cells appears more complex (Knobloch *et al.,* 1988). Although a similar water solubility tendency is observed, specific statements on the action of single terpenoids *in vivo* have to be assessed singularly, taking into account not only the structure of the terpenoid but also the chemical composition of the cell wall (Knobloch *et al.,* 1988). Despite a lack of mechanistic understanding, these plant extracts clearly demonstrate antibacterial properties. These activities suggest potential use as chemotherapeutic agents, food preserving agents and disinfectants.

Chemotherapeutic agents used orally or systemically for the treatment of microbial infections of humans and animals possess varying degrees of selective toxicity. Although the principle of selective toxicity is used in agriculture, pharmacology and diagnostic microbiology, its most dramatic application is the systemic chemotherapy of infectious disease. The tested plant products appear to be effective against a wide spectrum of microorganisms, both pathogenic and non-pathogenic. Administered orally, these compounds may cause an imbalance in the gut microflora, possibly allowing opportunistic pathogenic coliforms to become established in the gastrointestinal tract resulting in serious effects upon health. Furthermore, they may exert unacceptable cytotoxic effects upon healthy cells, e.g. eugenol and clove oil used in dentistry produce side effects such as contact dermatitis (Wade, 1977) and severe burning of the oral mucosa (Lavelle and Proctor, 1978). Consequently, their usefulness may be limited to topical applications, where *in vitro* activity would be expected to be reproduced *in vivo*. Further studies on therapeutic applications of volatile oils should be undertaken to investigate these issues, especially when considering the vast amount of analytical studies carried on these natural products.

As disinfecting agents, the volatile oils and their component volatility and lack of solubility make these plant extracts less appealing for general disinfectant

applications. They are poorly soluble in aqueous media and many are highly volatile at room temperature. However, a role as disinfectants of rooms has been reportedly investigated in a classical study (Kellner and Kober, 1954). Their volatility would be a distinct advantage in lowering microbial contamination in air and on difficult to reach surfaces. Although the minimum inhibitory concentrations for a selection of oils tested in a closed chamber were lower in the vapour phase (Inouye *et al.,* 1983), evidence suggests that such applications may have merit (Makarchuk *et al.,* 1981; Taldykin, 1979).

As food preservatives, volatile oils may have the most potential use. Spices, which are used as integral ingredients in cuisine or added as flavouring agents to foods, are present in insufficient quantities for their antimicrobial properties to be significant. However, spices are often contaminated with bacterial and fungal spores (Deans, personal communication) due to their volatile oil content, often with antimicrobial activity, being enclosed within oil glands and not being released onto the surface of the spice matter. Volatile oils, which often contain the principal aromatic and flavouring components of herbs and spices, if added to foodstuffs would cause no loss of organoleptic properties, would ameliorate microbial contamination and therefore retard spoilage. In addition, small quantities would be required for this effect. Furthermore, as described in Chapter 5 and numerous published reports, evidence suggests that these oils possess strong antioxidant properties also required to combat organoleptic deterioration and increase shelf life.

4. Antimycotic Effects of Selected Plant Volatile Oils Upon Three Fungal Organisms

4.1. Introduction

Screening of both crude and purified isolates from natural sources for antifungal activity has experienced resurgence. This resurgence is driven by the desire to supersede the efficacy of contemporary agents, the accusation that broad-spectrum antibiotics are causing a wider dissemination of already present fungal infection, the discovery of new lead compounds for further development, the substitution of synthetic agents used as preservatives by natural products and as a result of the increased awareness of phytotherapy. A major potential source of antimycotic agents would be expected from the plant kingdom and potentially from plant volatile oils.

Volatile oils extracted from aromatic and medicinal plants have a traditional role in many non-western cultures and itinerant populations predominately from the Mediterranean regions of Europe as a means of treating systemic and superficial mycoses and as preservatives against fungal growth and mycotoxin production in human foods and animal feeds. A plethora of published literature catalogue and confirms the beneficial properties of botanicals used as tradition remedies and illustrate the range of plant families and species capable of producing volatile oils, which have demonstrable antifungal properties. The fungistatic and fungicidal activity of plant volatile oils and their components have been investigated against a range of biologically significant fungi and yeasts including *Aspergillus flavus* (Sinha *et al.,* 1993), *A. ochraceus* (Deans *et al.,* 1995) and *A. niger* (Charai *et al.,* 1996; Mei-Chin *et al.,* 1997), *Candida albicans* (Mei-chin *et al.,* 1997), *Fusarium* species including *F. culmorum* (Deans *et al.,* 1995) and *Penicillium* species including *Pen. parasiticus* (Charai *et al.,* 1996; Qamar *et al.,* 1994).

In this investigation, two *Aspergillus* species and one *Fusarium* species were used in this study, Table 35. *Aspergillus* species, classified in the Deuteromycetes, are commonly associated with food spoilage and other materials. Significantly, *Aspergillus* species are capable of growth and metabolism at low water activities that

make foods too dry to be attacked by other microorganisms. *A. flavus* is a mycotoxin-producing filamentous fungus widely encountered as a contaminant of cereal crops and other foodstuffs especially in regions of the world with high humidity (Groopman *et al.,* 1988). It produces the mycotoxins known as aflatoxins including aflatoxin B_1 which is a potent hepatocarcinogen (Peers *et al.,* 1987) and aflatrem. *A. niger* is also a mycotoxin producing fungus, producing malformins and oxalic acid.

Fusarium species demonstrate a range of activities including specific plant pathogens to saprophytic on senescent plant materials to biodegradation of industrial products (Thomas, 1984). *Fusarium culmorum* (W.G. Smith 1884) is of the family *Hyphomycetes* and was previously classified in the Deuteromycetes but widely considered an anamorphic genus affiliated with Hypocreales (Ascomycetes). It is responsible for diseases such as ear rot in corn and head blight (van Wyk *et al.*, 1987) and scab in wheat (Calhoun and Park, 1964), causing significant economic damage to wheat farmers; production of the mycotoxins trichothecenes, toxic sesquiterpene metabolites, and zearalenones which are found in grains and grain products (Munkvold and Desjardins, 1997), human foods and animal feeds (Food Research Institute Briefings, 1997). Although the mycotoxins produced are not the most toxic, they are associated with illness in farm animals and humans (Bhat *et al.*, 1997) and are heat-stable and consequently ordinary cooking and procedures for heat processing do not substantially reduce toxin levels (Neira *et al.*, 1997). Despite intensive research, efforts to control *Fusarium* fungal infections and prevent or eliminate the presence of its mycotoxins in foods have not met with great success (Food Research Institute Briefings, 1997).

The aims of this investigation were as follows:

1. To screen the inhibitory effects of black pepper, clove, melissa, oregano and thyme volatile oils upon the growth of *Aspergillus flavus, Aspergillus niger* and *Fusarium culmorum* fungal species.
2. To determine which oils were the most effective volatile oils.

4.2. Materials and Methods

4.2.1. Materials

Potato Dextrose Agar [CM 139] (PDA) was purchase from Oxoid Unipath Ltd., Hampshire, UK.

4.2.2. Fungal Strains

Fungal strains (Table 35) were maintained on PDA at room temperature and were subcultured every month. Two weeks prior to testing. the cultures were checked for purity by plating out a loopful of the inoculated broth onto a PDA plate incubated at 25°C for two weeks which showed the presence of any contamination.

Table 35 Fungal strains and their source.

Organisms	Source	Classification
Aspergillus flavus	IMI 89717	Mycotoxigenic Spoilage
Aspergillus niger	IMI 17454	Common Spoilage
Fusarium culmorum	IMI 307847	Plant Pathogen

IMI: International Mycological Institute, Kew, Richmond, Surrey.

4.2.3. Assessment of Fungal Growth

Inhibition of fungal growth was measured using the method previously described by Deans and Svoboda (1990). Conidia from the fungal species were harvested in sterile distilled water containing 0.1% [v/v] Tween 80 (Koch-Light . washed five times in distilled water then stored at 5°C until used as inocula [final concentration of approximately 10^5 conidia mL^{-1}] for 50mL Erlenmeyer flasks. containing 25mL Yeast Extract Sucrose [YES] broth which had been autoclaved at 121°C for 25 minutes (Davies *et al.*, 1966). Inoculation of the flasks with the fungal suspension and the addition of the plant volatile oil was carried out under aseptic conditions. The experiment was carried out in triplicate.

The oil being tested was added to the flasks at a concentration range from 1 to 100µL mL^{-1} YES broth: three flasks received no sample and were used as controls.

All flasks were incubated on a shaking platform at 25°C, in the dark for seven days after which the mycelium was collected on GF/C filter papers [5.5cm diameter, Whatman]. The flasks were rinsed out three times with distilled water and the filter papers were then placed in a drying oven [105°C] overnight. The filter papers were weighed to constant weight, and the percentage of inhibition of growth relative to mean weight of mycelium from the control flasks was calculated using the following equation:

$$\frac{[C-T]x100}{C}$$

C= Dry weight of mycelium in control flask.

T= Dry weight of mycelium in test flasks.

4.2.4. Statistical Analysis

Statistical analysis was limited to the calculation of the mean of the measured values, in agreement with published data. All statistical analyses were carried out using Minitab® for Windows 32Bit Release 10.5 Xtra software.

4.3. Results

The growth inhibitory effects of the volatile oils from black pepper, clove, melissa, oregano and thyme at different concentrations on the filamentous fungi *Aspergillus flavus*, *Aspergillus niger* and *Fusarium culmorum* were evident at concentration levels tested (1μL mL^{-1}, 2μL mL^{-1}, 5μL mL^{-1}, 10μL mL^{-1}, 20μL mL^{-1}, 50μL mL^{-1} and 100μL mL^{-1} broth), Figure 23 to Figure 25.

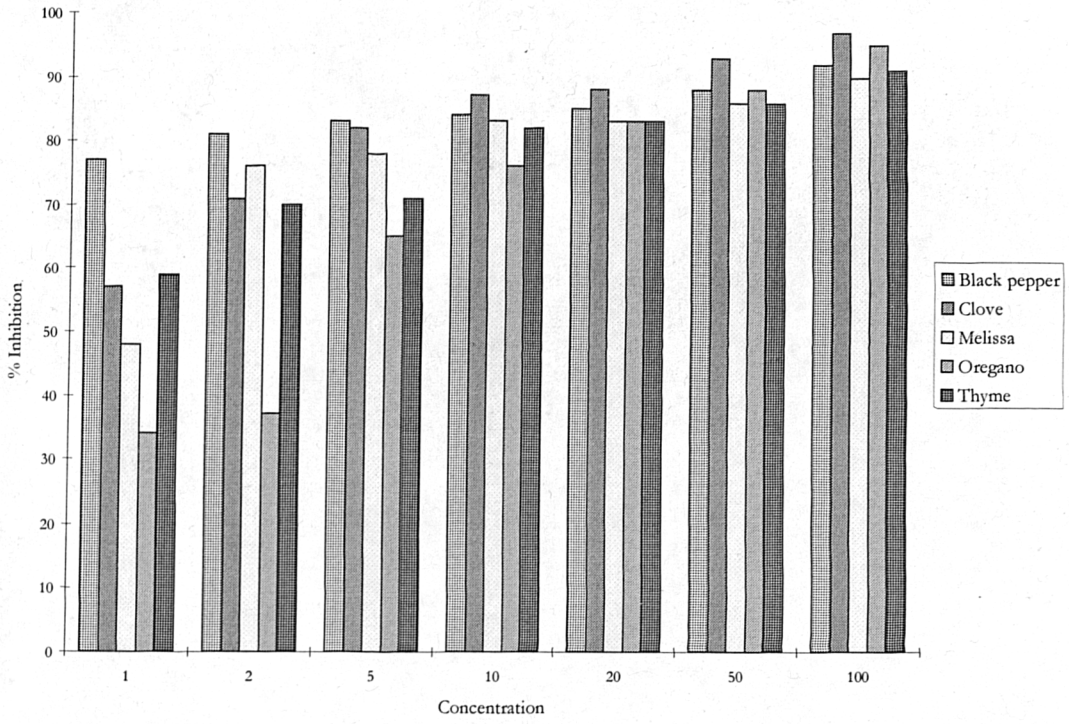


Figure 23. The antifungal activity of black pepper, clove, melissa, oregano and thyme volatile oils against *A. flavus*.

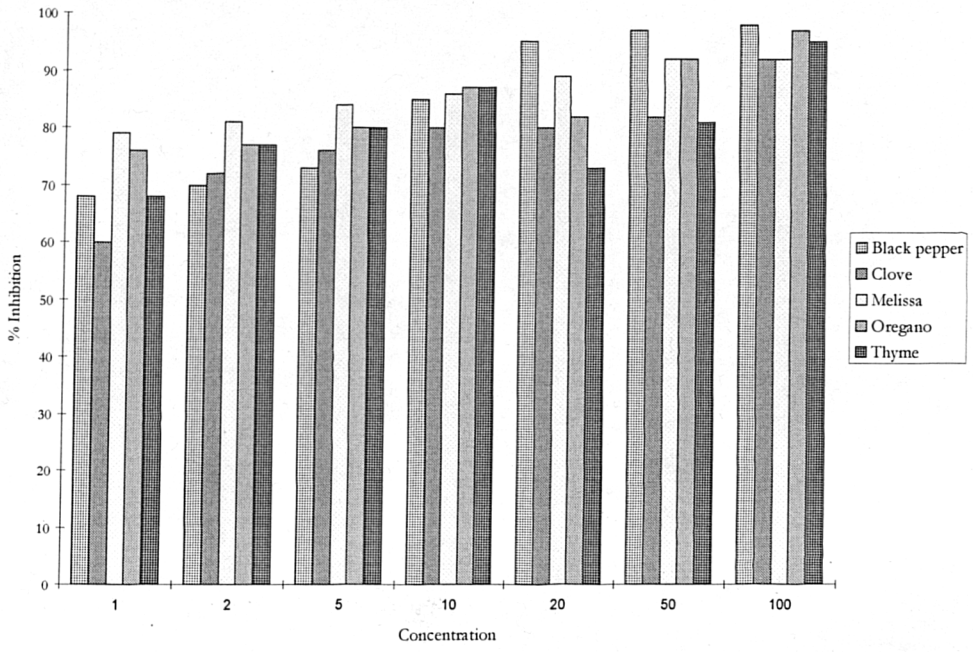


Figure 24. The antifungal activity of black pepper, clove, melissa, oregano and thyme volatile oils against *A. niger*.

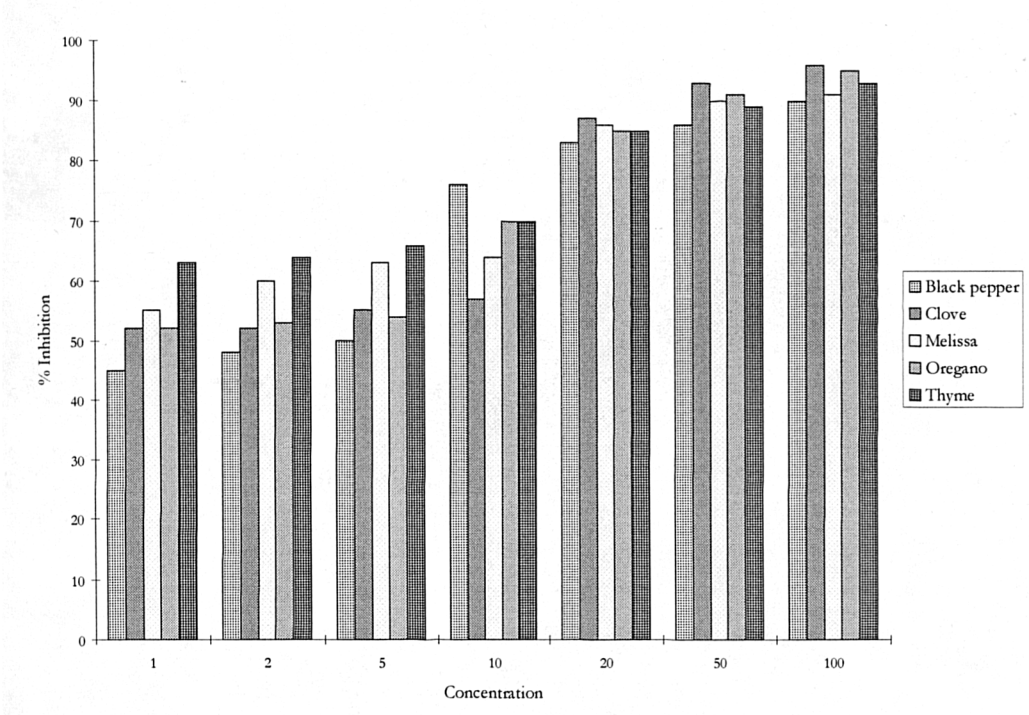


Figure 25. The antifungal activity of black pepper, clove, melissa, oregano and thyme volatile oils against *F. culmorum*.

4.3.1. Fungal Susceptibility

The two *Aspergillus* species and the *Fusarium* species all demonstrated variable growth inhibition when incubated in the presence of each volatile oil used in this study, as described in the Materials and Methods. In addition, the fungi were inhibited in a concentration-dependent fashion from the lowest volatile oil concentration level (1μL mL^{-1} broth) to the highest concentration level (100μL mL^{-1} broth) used. to the tested volatile oils particularly at the lower concentration ranges. As the concentration levels increased, this fungus demonstrated similar degrees of inhibition on its growth.

In the case of the fungus *A. flavus*, the % growth inhibition (mean±SD) for the tested volatile oils was calculated as 55.0±15.7% at 1μL mL^{-1}, 67.0±17.3% at 2μL mL^{-1}, and 75.8±7.7% at 5μL mL^{-1} while at the remaining concentrations 82.4±4.0% (10μL mL^{-1}), 84.4±2.2% (20μL mL^{-1}), 88.2±2.9% (50μL mL^{-1}) and 93.0±2.9% (100μL mL^{-1}). The high standard deviation at the concentration levels 1μL mL^{-1} to 2μL mL^{-1} is due to the relatively poor activity of the volatile oil of oregano at these

concentrations. At the remaining concentration levels, the activities of all the volatile oils were more uniform, especially at the highest treatment levels.

In the case of the fungus *A. niger*, the % growth inhibition for the tested volatile oils was more uniform than for the *Aspergillus flavus* species, with 70.2±7.5% at 1μL mL^{-1}, 75.4±4.4% at 2μL mL^{-1}, and 78.6±4.2% at 5μL mL^{-1} while at the remaining concentrations 85.0±2.9% (10μL mL^{-1}), 83.8±8.5% (20μL mL^{-1}), 88.8±7.0% (50μL mL^{-1}) and 94.8±2.8% (100μL mL^{-1}). The tested volatile oils were more consistently active at each concentration level against this *Aspergillus* species.

In the case of the fungus *F. culmorum*, the % growth inhibition for the tested volatile oils was 53.4±6.5% at 1μL mL^{-1}, 55.4±6.5% at 2μL mL^{-1}, and 57.6±6.7% at 5μL mL^{-1} while at the remaining concentrations 67.4±7.2% (10μL mL^{-1}), 85.2±1.5% (20μL mL^{-1}), 89.8±2.6% (50μL mL^{-1}) and 93.0±2.6% (100μL mL^{-1}).

4.3.2. Volatile Oil Activity

The volatile oil of black pepper was found to be particularly effective inhibitor of the growth of the *A. flavus* and *A. niger* species across the concentration range used and especially at the lower concentration levels, while this volatile oil was less effective at the lower concentration levels against *Fusarium culmorum*. Nevertheless, all these fungi showed marked inhibition over the investigated concentration levels of this oil. *A. flavus* was the most susceptible fungus to this volatile oil, especially at the at the 1μL mL^{-1} concentration level, with thyme volatile oil being the closest in terms of activity at 59% growth inhibition. In the case of *A. niger*, black pepper volatile oil was lonely slightly less effective against this particular species at the lower concentration levels (1μL mL^{-1} to 5μL mL^{-1}) but was more effective across the remaining treatment levels, relative to *A. flavus*. Although this volatile oil demonstrated % growth inhibition levels of 45%, 48% and 50% at 1μL mL^{-1}, 2μL mL^{-1}, 5μL mL^{-1} concentration levels respectively, these levels of activity were less than demonstrated against the two *Aspergillus* species at comparable concentrations.

Clove volatile oil inhibited the growth of all the fungal cultures. At concentration levels 1μL mL^{-1} through to 10μL mL^{-1}, this volatile oil was more

effective at inhibiting the growth of both the *Aspergillus* species than *F. culmorum*, while at the remaining concentration levels, *A. flavus* was only marginally more susceptible than *F. culmorum*.

The volatile oil of melissa was effective at inhibiting the growth of all fungal species used in this study. It was particularly active at all concentration levels tested against *A. niger*. 79% growth inhibition occurred at 1μL mL^{-1}, which increased to 81% growth inhibition occurred at 2μL mL^{-1} and similarly, only marginally increased across the remaining concentrations to 92% at 100μL mL^{-1} treatment level. The growth of *A. flavus* was less inhibited at 1μL mL^{-1}; however, at the 2μL mL^{-1} treatment level, 76% growth inhibition occurred. At the remaining concentration levels, the % growth inhibition gradually increased to a maximum of 90% growth inhibition. In the case of *F. culmorum*, melissa volatile oil inhibited 55% of this species growth relative to the untreated culture at 1μL mL^{-1} and gradually increased to 91% at 100μL mL^{-1}.

Oregano was most effective against *A. niger*, with little difference between the concentration ranges. 76% inhibition occurred at 1μL mL^{-1} broth, comparable to the activities expressed by melissa and thyme volatile oils. At the lowest two concentrations, oregano was poorly effective against *A. flavus* only inhibiting 34% and 37% at 1μL mL^{-1} and 2μL mL^{-1} broth respectively. Only at 10μL mL^{-1} broth did this oil match its activity at 1μL mL^{-1} broth against *A. niger*. This lack of activity was uncharacteristic of the other volatile oils tested against the *Aspergillus* species. As to why a relative lack of activity was found against *A. flavus* and not against *A. niger* is unclear. Against *F. culmorum*, there was little difference in inhibitory activity at concentrations 1μL mL^{-1} (52%), 2μL mL^{-1} (53%) and 5μL mL^{-1} (54%). However, at 10μLmL^{-1} this oil inhibited 70% growth relative to the untreated culture.

The volatile oil of thyme was highly active against all the fungal cultures used in this study, across the concentration range. The *Aspergillus* species were the most susceptible, being similarly susceptible at each concentration point. *F. culmorum* was only marginally less inhibited across the concentration range.

4.4. Discussion

4.4.1. Nature of Antifungal Activity

Regarding the nature of the antifungal activity, it seems most likely that the inhibitory effects of the volatile oils are due to their major components. However, the influence of minor or miscellaneous components may have a relatively important role due to possible undetermined synergistic interactions. A number of components have been documented as being active against different fungal strains. It is established that phenols are more effective than ketones which are more effective than oxides (Benjilali *et al.,* 1984; Kurata and Koike, 1982). Furthermore, monoterpenoic ketones have been reported as possessing almost the same effectiveness (Kurata and Koike, 1982).

Clove volatile oil is essentially entirely composed of the phenylpropanoid eugenol (Table 11), a constituent known to be more inhibitory than benzoic acid as early as 1911 (Hoffman and Evans, 1911). Eugenol has been demonstrated as highly inhibitory when tested against *Aspergillus* species, causing complete inhibition at 0.4mg mL^{-1} against *A. flavus* in culture media (Hitokoto *et al.,* 1980) and has been as the core source for the strong bioactivities of pimento and cinnamon leaf volatile oils (Lis-Balchin *et al.,* 1998).

The principal component in thyme oil is the phenolic substance thymol (48.00%), Table 21. Thymol and carvacrol are known to possess high antifungal properties (Benjilali *et al.,* 1984; Hitokoto *et al.,* 1980). The remaining components in this oil, *p*-cymene (25.52%), linalool (4.56%), γ-terpinene (4.44%), carvacrol (2.95%), α-pinene (2.12%), bornyl acetate (1.91%) and 1,8-cineole (1.25%) are recognized as possessing strong antifungal properties, notably *p*-cymene (Morris *et al.,* 1979; Piccaglia *et al.,* 1993; Reuveni *et al.,* 1984; Ross *et al.,* 1980), linalool (Maruzzella *et al.,* 1961; Morris *et al.,* 1979, Piccaglia *et al.,* 1993; Reuveni *et al.,* 1984), α-pinene (Morris *et al.,* 1979) and 1,8-cineole (Kurata and Koike, 1982; Morris *et al.,* 1979; Piccaglia *et al.,* 1993).

The mode of action of these components appears to be variable. Work carried out by Moleyar and Narasimham (1987a) suggests that the components citral (*cis+trans*) and camphor act by inhibiting the uptake of metabolites (glucose, acetate

and phenylalanine) by fungal hyphae, by inhibiting the biosynthesis of RNA, DNA and proteins and by binding to the lipid phase of the membrane thus altering its permeability.

4.5. Conclusion

The results presented in this study demonstrate that these plant volatile oils have value as naturally derived fungicidal agents and highlight the possibility that plant volatile oils may have an important role in crop protection (Arras and Picci, 1984; Maruzzella and Balter, 1959) and in the preservation of foodstuffs against filamentous fungi (Baratta *et al.,* 1998a; Baratta *et al.,* 1998b; Baratta *et al.,* 1998c). Furthermore, natural compounds with significant antifungal properties may provide novel pharmaceutical agents to combat mycoses and mycotoxicoses which pose an increasingly significant threat to public health, compounded by rising antibiotic resistance and HIV infection.

5. Antioxidant Properties of Plant Volatile Oils: An *In Vitro* Assessment

5.1. Introduction

Lipid peroxidation is a paradox of aerobic life affecting both human health and the quality of modern life. Practically everything used from cosmetics and pharmaceuticals to foodstuffs are susceptible to oxidative damage unless protected. Biological systems, rich in lipids, are susceptible to autoxidation unless protected by either endogenous mechanisms, e.g. enzymes, vitamins or miscellaneous cellular constituents or through the diet. In certain circumstances, such as ageing, chemical insult, dietary deficiencies or disease states, it is necessary to augment the natural defense mechanisms such as against uncontrolled peroxidative attack.

Numerous compounds synthesised by chemists have been very successful in retarding potential oxidative damage in susceptible media. However, the toxicology of a number of these compounds, e.g. BHA and BHT is confused, leading to concerns regarding their safety in nutritional products for human consumption (Iverson, 1995; Jayalakshmi and Sharma, 1986; Schilderman *et al.*, 1995; Thompson and Trush, 1988a; Thompson and Trush, 1988b; Tobe *et al.*, 1986).

Although the toxicity demonstrated for these compounds may only be of evolutionary significance, these concerns have stimulated the search for and the utilization of plant, marine and microbial sourced compounds with antioxidant properties which, perceived as safer, are more appealing to the public as food additives and nutraceuticals. Traditionally, the medicinal use of plant volatile oils from aromatic and medicinal plants has stemmed from their antibacterial and antifungal activities. Recently, however, their antioxidant properties been investigated and their potential evaluated (Simpson, 1995; Youdim, 1997).

The aim of this study was to screen *in vitro* the ability of the plant volatile oils of black pepper, clove, geranium, melissa, monarda, nutmeg, oregano and thyme to retard the onset of lipid peroxidation, identify components responsible for such activity and determine their mechanism of action. This was undertaken using a modified thiobarbituric acid reactive species (TBARS) assay (Baratta *et al.*, 1998a;

Baratta *et al.*, 1998b; Baratta *et al.*, 1998c; Dorman *et al.*, 1995a), a β-carotene agar diffusion assay (Araujo and Pratt, 1985), a conjugated diene assay (Foti *et al.*, 1996) and a radical trapping (TRAP) assay (Miller *et al.*, 1993a; Rice-Evans and Miller, 1994).

5.2. Materials and Methods

5.2.1. β-Carotene Antioxidant Assay

The β-carotene agar diffusion assay has been proposed as a method for the rapid measurement of lipid antioxidant activity of specific compounds or combination of compounds (Araujo and Pratt, 1985) and has advantages over alternative assays utilizing β-carotene (Miller *et al.*, 1993; Pratt and Watts, 1964). The retention of the colour of β-carotene has been proposed as being very useful in measuring antioxidant activity in an oxidising system composed of fat or fatty acids in the presence of antioxidants. This method allows for the rapid screening of a large number of samples, the screening of compounds which impart colour or form turbid alcoholic solutions which make spectral readings difficult (Araujo and Pratt, 1985).

The β-carotene agar diffusion technique is essentially that of Araujo and Pratt (1985) whereby wells are punched in agar plates containing β-carotene and linoleic acid into which is placed 15μL of the test sample. The development of a yellow colour surrounding the deposit of the test agent demonstrates antioxidant activity. The intensity and/or persistence of colour are proportional to its antioxidant capacity.

5.2.1.1. Materials

β-Carotene and linolenic acid were purchased from Sigma Chemical Co., Poole, Dorset, England. Bacto-agar was purchased from Oxoid Unipath Ltd., Hampshire, UK. The plant volatile oil components were purchase either from Sigma Chemical Co., Poole, Dorest, England or Fluka Fine Chemicals, Gillingham, Dorest, England.

5.2.1.2. Experimental Procedure

Diffusion plates used to measure the potential antioxidant capacities of the volatile oils and their constituent components were prepared as follows:

1. 2.0g agar (Bacto-agar) was dissolved in 100mL of boiling water.

2. The agar solution was allowed to cool to approximately 50°C, then 2.0mL of linoleic acid in ethanol (2.0mg mL^{-1}) and 10mL of β-carotene in acetone (2.0mg mL^{-1}) was flushed into the agar.

3. The agar was poured into Petri dishes and allowed to harden for 30 minutes. As acetone is used to solubilize the β-carotene, the agar does not completely set hard but remains jelly-like.

4. Two holes (4mm diameter) were punched into the agar of each Petri dish.

5. Into each hole was added 15μL of test sample.

6. Plates with test samples were incubated at 45°C overnight until the background colour had bleached.

7. The surrounding zones of yellow colour around the test sample wells were marked by pen and the diameter of the circle measured using Vernier calipers.

5.2.1.3. Statistical Analysis

Statistical analysis was limited to the calculation of the mean of the measured values and the standard error of the mean (S.E.M.) in agreement with published data. All statistical analysis was carried out using Minitab® for Windows 32Bit Release 10.5 Xtra software.

5.2.2. Thiobarbituric Acid Reactive Species (TBARS) Assay

The thiobarbituric acid assay is a well established assay system (Bernheim *et al.,* 1948; Kikugawa *et al.,* 1992; Kosugi *et al.,* 1988; Kosugi *et al.,* 1989; Ohkawa *et al.,* 1978; Ohkawa *et al.,* 1979) and most frequently used for the measurement of the occurrence of lipid peroxidation both *in vivo* and *in vitro* inspite of numerous warnings of the limitations of the test or the uncertainties in the identification of the TBA-reactive species and the danger of using TBA numbers as indices of

autoxidation. Numerous variations in sample preparation and methodology have been reported (Botsoglou *et al.,* 1994; Dorman *et al.,* 1995a; Ke *et al.,* 1984; Rhee, 1978). This methodology is capable of measuring peroxidative degradation of fatty acids in complex biological and food systems (Kikugawa *et al.,* 1992; Know *et al.,* 1983; Kosugi *et al.,* 1989; Moerck and Ball, 1974; Pikul *et al.,* 1984). The assay relies upon the reaction of malondialdehyde, the split product of an endoperoxide of unsaturated fatty acids (Figure 4), with thiobarbituric acid on heating under acidic conditions, Figure 26. The reaction product 1:2 MDA-TBA is a pink adduct which can be measured photometrically at 532nm after solvent extraction.

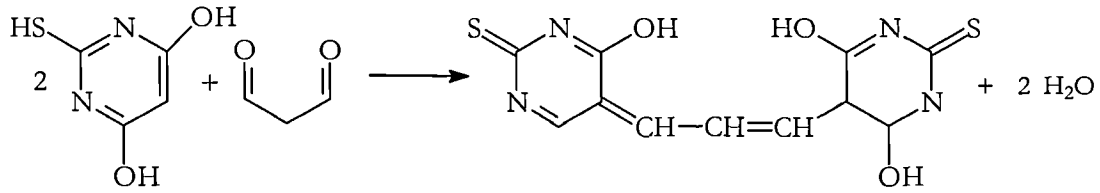


Figure 26. The chemical reaction of MDA with TBA under acid conditions.

5.2.2.1. Materials

Thiobarbituric Acid was purchased from Sigma Chemical Co., Poole, Dorset, England. The volatile oil components were purchase either from Sigma Chemical Co. or Fluka Fine Chemicals, Gillingham, Dorest, England. Ethanol, methanol, butan-1-ol and acetic acid were purchase from BDH, Lutterworth, England. Potassium chloride and sodium hydroxide were purchased from Aldrich. The egg yolk, livers and muscle samples were obtained from commercial suppliers or the Department of Poultry Science, Scottish Agricultural College, Ayr, Scotland. Rat liver samples were obtained from the Department of Biological Sciences, Newcastle University, Newcastle, England.

5.2.2.2. Sample Preparation

All sample material tested was tested in a number of vehicle solutions for solubility. Ethanol (pure) or glass-distilled methanol were used as the solvents to dissolve the sample material unless used neat. By diluting the test samples appropriately, ranges of concentrations were produced.

5.2.2.3. Substrate Preparation

Egg yolk was used as a lipid rich substrate for this assay. The egg sac was separated from the albumin and placed on filter paper. An incision was made into the yolk sac and the yolk material was removed, leaving behind the sac membrane. After an appropriate amount of yolk was obtained, it was mixed vigorously and placed under nitrogen into a sealed glass container and kept at -20°C until required. Homogenates (10% w/v) were prepared immediately prior to use with KCl (1.15% w/v) using a Ystral T1500 tissue homogenizer for 30 seconds. After homogenization, the yolk sample was ultrasonicated using a F52006 ultrasonic bath (Decon Laboratories Ltd., Hove, Sussex, UK) for three minutes then poured into a test tube.

5.2.2.4. Preparation of Solutions

The potassium chloride solution was prepared by dissolving 1.15g KCl in 100mL distilled water. The sodium dodecyl sulphate/thiobarbituric acid solution was prepared by adding either 8.10g (or 1.10g depending upon the required concentration needed) to 100mL distilled water which was then gently heated and stirred using a magnetic flea on a heating plate until any solids were completely dissolved; to this was added 0.80g thiobarbituric acid. This solution was prepared immediately prior to use and maintained on the heating plate and continuously stirred until used so as to prevent TBA material precipitating out of solution. Acetic Acid (20% v/v) pH 3.5 [200mL] was prepared by adding 40mL acetic acid and 65mL 1M sodium hydroxide to approximately 95mL distilled water, with the pH being finely tuned by adding single drops of 1M sodium hydroxide solution.

5.2.2.5. Experimental Procedure

Add 0.5mL egg yolk homogenate (10% w/v) to each test tube. To this add 0.1mL of test oil or component to be tested, dissolved in an appropriate solvent vehicle if solid or to a specific concentration. Briefly vortex until well mixed (5 seconds). Make up to 1.0mL volume with distilled water. To this add 1.5mL acetic acid (20% v/v), vortex; add 1.5mL TBA (0.8% w/v) solution then vortex.

Place test tubes into a heating block set at 95°C, using glass balls as stoppers to prevent evaporation, and leave for a period of 60 minutes. After this period of time, place into cold water for 5 minutes to stop any further reactions. Add 5.0mL

butan-1-ol and shake. Centrifuge the mixed solutions at 1500g for a period of 10 minutes to form two separate layers. The top layer was removed and pipetted into plastic cuvettes and the absorbance measured using a Pye Unicam SP8-500 UV/VIS (Cambridge) spectrophotometer, wave length set at 532nm.

5.2.2.6. Statistical Analysis

Statistical analysis was limited to the calculation of the mean of the measured values and the standard error of the mean (S.E.M.) in agreement with published data. All statistical analysis was carried out using Minitab® for Windows 32Bit Release 10.5 Xtra software.

5.2.3. Spectrophotometric Conjugated Diene Assay

The conjugated diene assay is a rapid and convenient method for determining the antioxidant efficiency of a test sample relative to α-tocopherol (RAE) based on the rate of oxidation of the fatty acid linoleic acid to its conjugate. This spectrophotometric assay relies upon the determination of the rate of generation of conjugated diene hydroperoxides from the peroxidative breakdown of linoleic acid induced by an azo-initiator initially in the presence of α-tocopherol and subsequently in the presence of a test compound in an aqueous SDS micellar system (Foti *et al.*, 1996; Pryor *et al.*, 1993). The azo-polymerizing agent 2,2'-azo*bis* (2-amidinopropane) dihydrochloride (ABAP), Figure 27, undergoes thermolytic decomposition to generate radicals at a steady rate (Pryor *et al.*, 1993). In the presence of this aqueous radical initiator, linoleic acid decomposes, producing conjugated diene hydroperoxides in the micellar phase, Figure 28. These hydroperoxides have a strong UV-absorption with a maximum at 234nm in SDS/NaH_2PO_4 buffered micelles, adjusted to pH 7.4 with 1M sodium hydroxide. This method has been reported to be extremely sensitive and allows the study of the processes of oxidation at low conversion rates, where the steady state kinetic analysis applies better (Foti *et al.*, 1996; Pryor *et al.*, 1993). Consequently, very small amounts of test sample are required.

The ratio of the slope of α-tocopherol over the slope of the suspected sample gives the RAE value for the latter, providing the experimental conditions are identical for both sets of experiments.

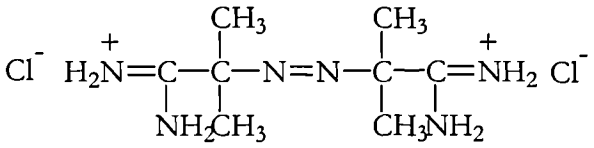


Figure 27. The water soluble azo initiator 2,2'azo*bis*(2-amidinopropane) dihydrochloride (ABAP). (Halliwell and Gutteridge, 1989).

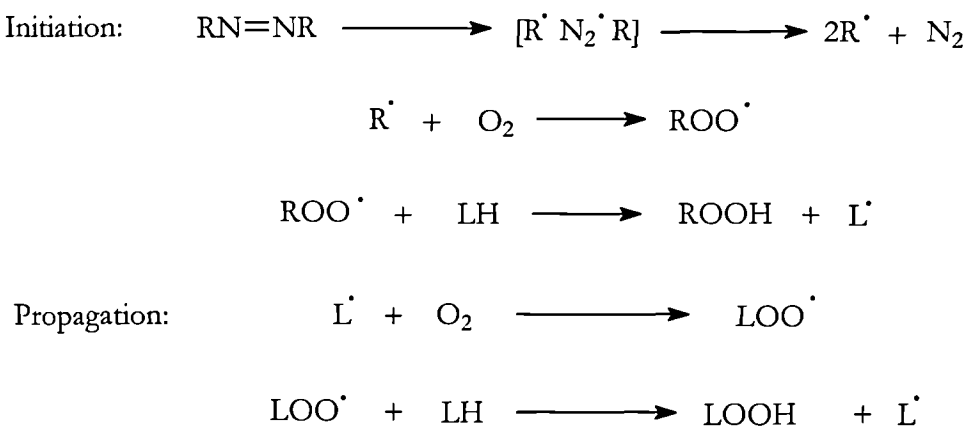


Figure 28. The mechanism of autoxidation of linoleic acid initiated with an azo initiator compound. Adapted from Pryor *et al.*, 1993.

Although the water-soluble initiator ABAP was used in this study, lipid soluble initiators such as 2,2'-azo*bis*(2,4-dimethylvaleronitrile) (DMVN), Figure 29, have also been used.

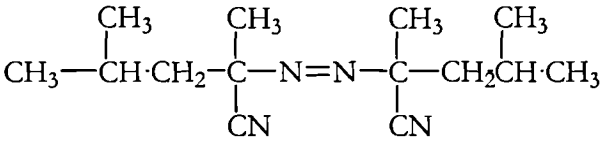


Figure 29. The lipid soluble azo initiator 2,2'-azo*bis* (2,4-dimethylvaleronitrile). (Halliwell and Gutteridge, 1989).

5.2.3.1. Materials

Linoleic acid and sodium dodecyl sulphate were purchased from Sigma Chemical Co., Poole, Dorset, England. 2,2'-azo*bis* (2-amidinopropane) dihydrochloride was a gift from Walko, Hamburg, Germany. Kinetics of the peroxidation process was measured using a Pye Unicam SP8-500 UV/VIS (Cambridge) spectrophotometer, equipped with a thermally controllable cuvette holder.

5.2.3.2. Solution Preparation

A 0.1M micelle suspension of sodium dodecyl sulphate (SDS) was prepared in aqueous 0.01M NaH_2PO_4, pH 7.4 adjusted using concentrated sodium hydroxide. To this suspension, linoleic acid to a concentration of 0.0026M was added immediately prior to use. A stock solution of 0.07M 2,2'-azo*bis* (2-amidinopropane) dihydrochloride was prepared in distilled water and stored at 5-10°C until required. This solution is stable at this temperature for 1 week. Solutions of the test compounds were prepared immediately prior to use in glass distilled methanol.

5.2.3.3. Experimental Procedure

A 2.0mL aliquot of the micelle suspension of linoleic acid in a UV-cell was stirred and placed in a sample compartment of a spectrophotometer set at 50°C. A SDS solution was used as a blank. After a 20 minute thermal equilibrium period, 10μL of the radical inducer solution was added and the progress of the peroxidation was recorded as absorbance at 234nm for a period of 15 minutes.

After this period, a number of volatile oils and components found in these oils were then added in order to screen their ability to prevent further production of conjugated dienes over a 30 minute period. Oils and components, which demonstrated activity, were then added in scalar amounts (10-50μL). The slope of the linear plot of absorbance *vs* time after the addition of the test compound gives dA/dt. Form the plot of dA/dt *vs* $[InH]^{-1}$, the slope S_{inh} was obtained and the RAE value calculated.

5.2.3.4. Statistical Analysis

Statistical analysis was limited to regression analysis in agreement with published data. All statistical analysis was carried out using Minitab® for Windows 32Bit Release 10.5 Xtra software.

5.2.4. Total Antioxidant Activity (Radical TRAP) Assay

The radical trapping (TRAP) assay is an established methodology for assessing a compounds ability to trap radicals before being able to participate in initiation or propagation stages of lipid peroxidation (Miller *et al.*, 1993; Rice-Evans and Miller, 1994). The TRAP method is based on the inhibition by antioxidants of the reaction of the ferryl-myoglobin radical species with 2,2'-azino*bis* (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) which after conversion into the radical cation ($ABTS^{+}$) has a specific maximum of the absorbance at 734 nm. The α-tocopherol analogue Trolox (2.5 mM) is used as a standard and the activities of the tested compounds are expressed as Trolox equivalents - the millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to 1.0mM solution of the substance under investigation.

5.2.4.1. Materials

Metmyoglobin (equine), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium ferricyanide and the buffer salts were purchased from Sigma Chemical Co., Poole, Dorset, England. 2,2' - azino*bis* (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and hydrogen peroxide from were purchased from Aldrich.

5.2.4.2. Solution Preparation

A 2.5mM Trolox solution was prepared by dissolving 0.156g of Trolox in 250mL PBS. At this pH the solution is near the upper limit of Trolox solubility. Gentile ultrasonification was required to dissolve the crystals. Trolox is stable when frozen for more than 6 months. Working concentrations of H_2O_2 were prepared from a stock solution of 500mM in PBS, 5mM ABTS was prepared by dissolving 0.0274g

of ABTS in 10.0mL of PBS buffer. ABTS was dissolved in this way has a millimolar extinction coefficient (ε_{mM}) of 38.8 at 340nm.

Metmyoglobin was purified prior to use on a Sephadex column in phosphate-buffered saline, pH 7.4. The concentration of myoglobin in the column elucate is calculated from the extinction coefficients, and aliquots of metmyoglobin in PBS were stored frozen until required for use. A 400μM solution of metmyoglobin (0.0752g in 10mL of PBS) was prepared; 0.0244g of potassium ferricyanide was dissolved in 100mL of PBS. 10mL of the ferricyanide solution was then added to 10mL of metmyoglobin solution. This mixture was then applied to the column and eluted with PBS. The first fraction was collected and its absorbance was read at 490, 560, 580 and 700 nm. The reading at 700nm was subtracted from the readings at 490, 560 and 580nm to correct for background absorbance. Calculation of the relative proportions of the different forms of myoglobin can be carried out using deconvolution procedures or by applying the Whitburn algorithms based on the extinction coefficients at 490, 560 and 580 nm.

The myoglobin prepared in this way should only be used if it constitutes more than 94% of the total haeme species present. The purified myoglobin is diluted with buffer to a concentration of 140μM if necessary, divided into aliquots and then stored frozen until required.

5.2.4.3. Experimental Procedure

A volume of PBS buffer was placed into a water bath set at 40°C and allowed to equilibrate. All other reagents were maintained at 4°C in ice throughout the experimental procedure. Hydrogen peroxide and the test samples were prepared fresh on each occasion.

Into a cuvette was added 489μL buffer, 10μL test sample, 300μL ATBS, 36μL metmyoglobin and 167μL H_2O_2. This was then thoroughly mixed and placed into the thematically controlled cuvette holder at 40°C. The absorbance was measured at 734nm for a period totaling 190 seconds using a Pye Unicam SP8-500 UV/VIS (Cambridge) spectrophotometer.

5.2.4.4. Statistical Analysis

Statistical analysis was limited to the calculation of the mean of the measured values and the standard error of the mean (S.E.M.) in agreement with published data. All statistical analyses were carried out using Minitab® for Windows 32Bit Release 10.5 Xtra software.

5.3. Results

5.3.1. β-Carotene Antioxidant Assay

The antioxidant potential of black pepper, clove, geranium, melissa, monarda, nutmeg and oregano volatile oils were tested using the β-carotene agar diffusion assay. All the oils demonstrated a degree of activity in this assay system (Table 36). The relative antioxidant capacity of the oils was found to be black pepper > melissa > monarda > oregano > geranium > clove > nutmeg. Black pepper and melissa were the most significantly active oil tested by this method. The oils of monarda, oregano, geranium and clove volatile oils expressed very similar activity in this assay. Nutmeg expressed significantly lower activity than the other oils. In order to assess which components may be responsible for the activity of these oils and their differences in activity, a number of their phytoconstituents were screened, as shown in Table 37.

5.3.1.1. Black Pepper

Black pepper volatile oil was determined to contain the main components β-caryophyllene (24.21%), limonene (11.51%), δ-3-carene (8.53%), β-pinene (7.33%), α-pinene (6.33%), eugenol (5.56%), α-terpineol (4.74%), α-phellandrene (2.74%), α-copaene (2.26%), α-humulene (2.16%), *p*-cymene (1.63%), γ-terpineol (1.48%) and phenyl methyl ester (1.23%), Table 10.

Neither β-caryophyllene, limonene, δ-3-carene, β-pinene, α-pinene, α-phellandrene, α-humulene nor *p*-cymene demonstrated activity in this assay. The only main component tested which demonstrated activity was eugenol. However, the activity of eugenol can not exclusively explain the activity of black pepper.

Table 36. Zone diameter of antioxidant protection (mm) of volatile oils in the β-carotene agar diffusion assay.

Volatile Oil	Diameter (mm)
Black Pepper	21.82±0.44
Clove	15.55±0.55
Geranium	16.05±0.45
Melissa	20.00±1.00
Monarda	17.65±0.35
Nutmeg	12.64±1.64
Oregano	16.65±0.35

All values are represented as the mean±S.E.M. for three independent experiments.

5.3.1.2. Clove

Clove volatile oil was determined as containing the main components eugenol (86.48%), β-caryophyllene (5.58%), eugenyl acetate (5.15%), α-humulene (0.66%), β-caryophyllene oxide (0.32%) and methyl salicylate (0.28%), Table 11. From the data presented in Table 37, the activity of clove volatile oil is derived from its major constituent eugenol. Neither β-caryophyllene nor α-humulene demonstrated any activity, while eugenol possessed significant activity (16.20±1.10mm). It was not possible to assess the activities of the remaining components: eugenyl acetate, caryophyllene oxide or methyl salicylate as they were not commercially available.

5.3.1.3. Geranium

Geranium oil was determined as containing the main components citronellal (32.92%), *trans*-geraniol (11.95%), citronellyl formate (7.55%), linalool (6.62%), *iso*-menthone (5.83%), menthone (1.75%), β-caryophyllene (1.20%) and geranyl tiglate (1.12%), Table 12. Of the components test present in geranium none of these compounds could explain the activity of this volatile oil.

5.3.1.4. Monarda

Monarda oil was determined as containing the main components thymol (70.59%), *p*-cymene (10.63%), carvacrol (6.12%) and terpinen-4-ol (1.23%), Table 17. Both the components carvacrol and thymol had high antioxidant properties while

the remaining compounds demonstrated no activity. The activity of monarda most probably lies with these two components.

5.3.1.5. Nutmeg

Nutmeg oil was determined as containing the main components α-pinene (22.04%), α-pinene (21.53%), sabinene (15.39%), myristicin (9.43%), terpinen-4-ol (5.72%), limonene (3.87%), myrcene (1.89%), *p*-cymene (1.87%), γ-terpinene (1.80%), α-terpinene (1.24%) and α-thujene (1.18%), Tabe 18. None of the main components tested demonstrated any activity in this assay. This suggests that relative lack of activity for nutmeg volatile oil is due to the lack of main component with antioxidant properties or is due to an undetermined synergist effect.

Table 37. Zone diameter of antioxidant protection (mm) of volatile oil components in the β-carotene agar diffusion assay.

Volatile Oil Components	Diameter (mm)
β-Bisabolene	0
Borneol	0
Camphene	0
δ-3-Carene	0
Carvacrol	11.45±0.35
β-*trans*-Caryophyllene	0
p-Cymene	0
Eugenol	16.20±1.10
α-Humulene	0
R (+)-Limonene	0
Linalool	0
Myrcene	0
trans-β-Ocimene	0
α-Phellandrene	0
α-Pinene	0
β-Pinene	0
Sabinene	0
Terpinen-4-ol	0
α-Terpinene	0
γ-Terpinene	0
Terpinolene	8.10±0.10

All values are represented as the mean±S.E.M. for three independent experiments.

5.3.1.6. Oregano

Oregano was determined as containing the main components carvacrol (69.37%), *p*-cymene (12.70%), γ-terpinene (2.09%), myrcene (1.45%), thymol (1.36%) and α-terpinene (1.01%), Table 20. The activity of oregano is most likely to responsible to both carvacrol and thymol.

The lack of activity demonstrated by the majority components in this assay is very interesting in comparison to the activities demonstrated by the volatile oils. Based upon the data generated from this assay alone, one might conclude that the activities of the volatile oils were a result of minor components not tested, non-terpenoid and a consequence of some undetermined synergistic relationship. However, when these components were tested in subsequent assays they appeared to demonstrate some degree of activity.

An explanation for the data is that the β-carotene assay may be an inappropriate assay for the assessment of volatile oils or their components for antioxidant activity. Agar, composed of polysaccharides, agarose and agar pectin is extensively used as a solidifying agent for microbiological studies. However, agar has been reported to reduce the activity of antimicrobial compounds such as phenols, antibiotics and metal salts by binding these substances, slowing their diffusion and hence reducing their activity (Moleyar and Narasimham, 1987b). Due to their physical and chemical properties, the components possibly generated false negatives in the β-carotene assay. By their nature, these compounds are very volatile. It is known from antimicrobial studies that the composition of volatile oils in the liquid phase change over time and enter a vapour phase above the test medium. Thus, some components weakly represented in the liquid phase are more important in the vapour phase. Components in oils and as authentic samples which enter the vapour phase would reduce the test materials ability to inhibit the oxidation of β-carotene especially when the number of antioxidant compounds are small either in composition or variety.

Alternatively, the assay lacks sufficient sensitivity to differentiate between the components tested. This demonstrates the importance in the screening of plant material for bioactivity to use a bank of assays *in vitro* before assigning bioactivities. By the use of a number of assays not only the number of false positives and negatives

should be greatly reduced but also evidence pertaining to the mechanism of action may be obtained.

5.3.2. Thiobarbituric Acid Reactive Species (TBARS)

5.3.2.1. Antioxidant Activities of α-Tocopherol and the Synthetic Antioxidants BHA, BHT and Pyrogallol in Egg Yolk, Chick Liver and Chicken Muscle Assays

BHA, BHT and pyrogallol are extensively used to retard peroxidative damage in susceptible systems, while α-tocopherol, present in both animal and plants, is an important part in the natural defense against radicals generated by UV radiation and xenobiotic metabolism. Their respective modes of action have been determined both *in vitro* and *in vivo*. These compounds were used to validate the modified TBARS assay (Dorman *et al.*, 1995a), based on three avian models to differentiate between the antioxidant capacity of test compounds both in the absence and presence of ferrous iron and ABAP inducers of lipid peroxidation.

Their ability to inhibit the generation of thiobarbituric acid species in the absence of lipid peroxidation in the egg yolk, chick liver and chicken muscle media are shown in Figure 30 to Figure 32. From the presented data, it is clear that the avian models were capable of demonstrating the antioxidant capacities of these antioxidant agents and differentiating between each compound in a dose-dependent manor. When tested in the presence of 1mM ferrous iron solution, the relative activities in the tested compounds were further enhanced in the egg yolk medium, Figure 33. This was also the case in the presence of the water soluble azo-polymerisation initiator ABAP, Figure 34.

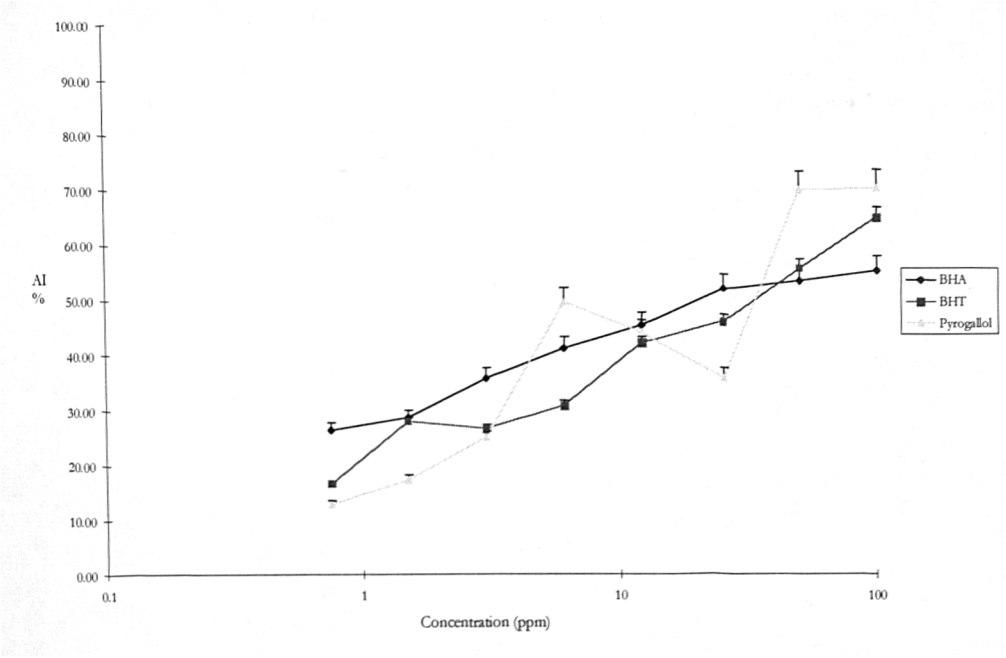


Figure 30. The antioxidant index (AI%) values for the synthetic antioxidants BHA, BHT and Pyrogallol as measured in the egg yolk based TBARS assay.

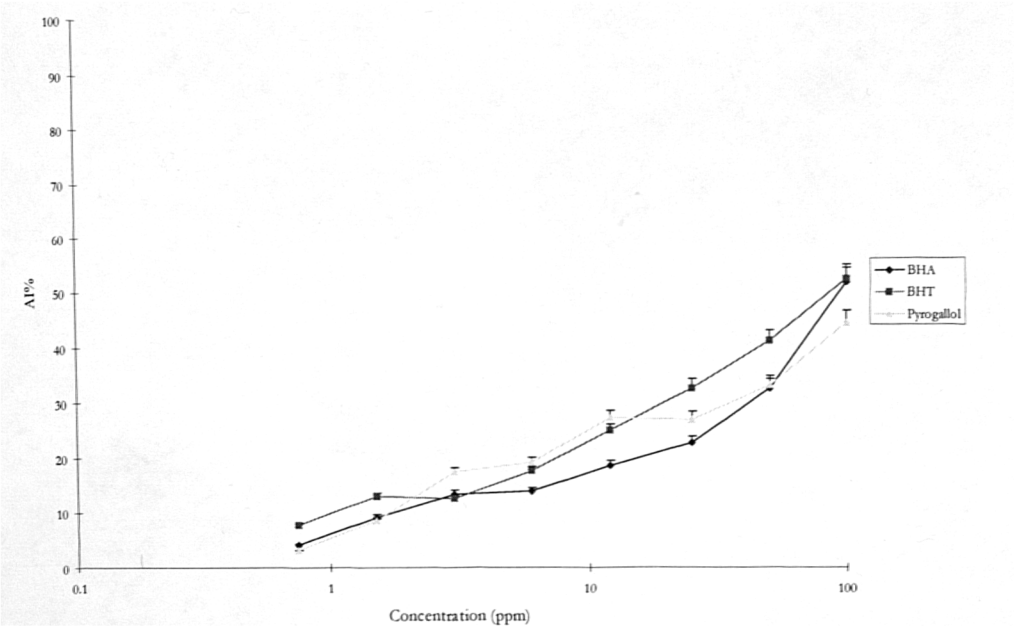


Figure 31. The antioxidant index (AI%) values for the synthetic antioxidants BHA, BHT and Pyrogallol as measured in the liver based TBARS assay.

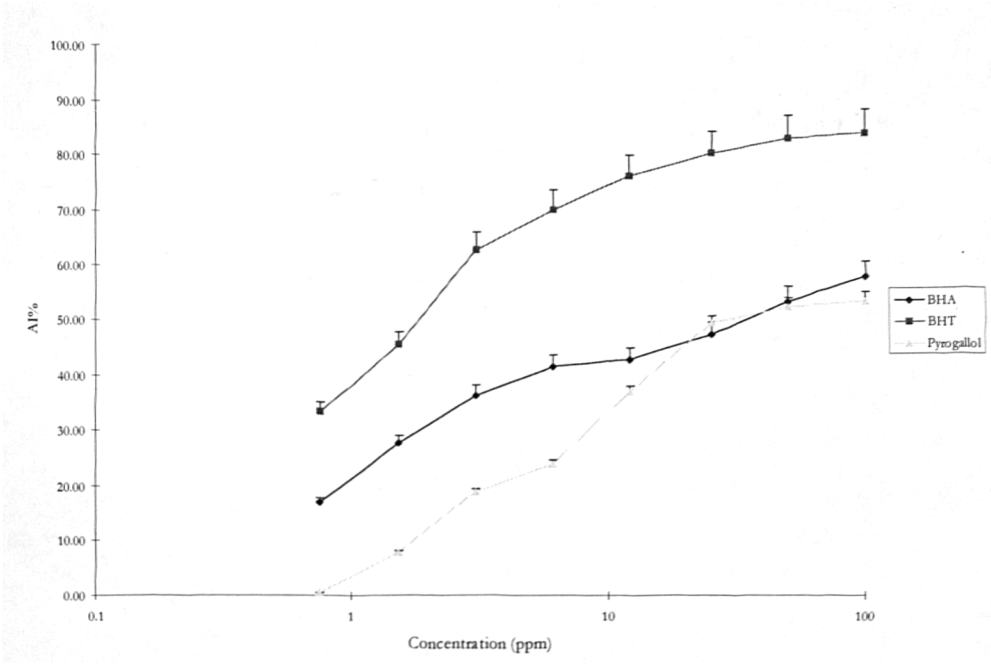


Figure 32. The antioxidant index (AI%) values for the synthetic antioxidants BHA, BHT and Pyrogallol as measured in the muscle based TBARS assay.

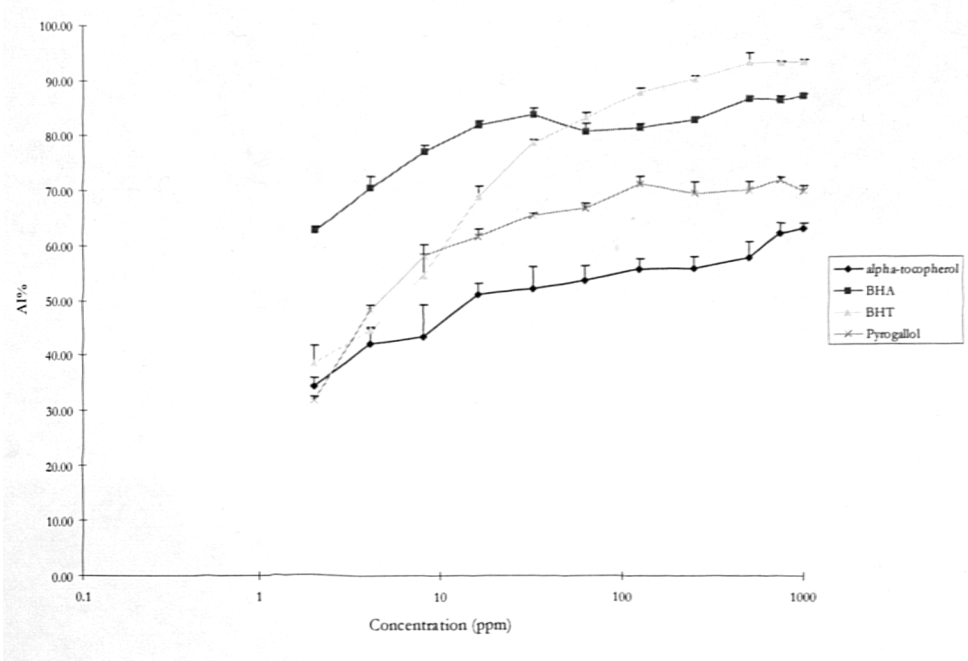


Figure 33. The antioxidant index (AI%) values for α-tocopherol and the synthetic antioxidants BHA, BHT and pyrogallol in the presence of 1mM $FeSO_4$ as measured in the egg yolk TBARS assay.

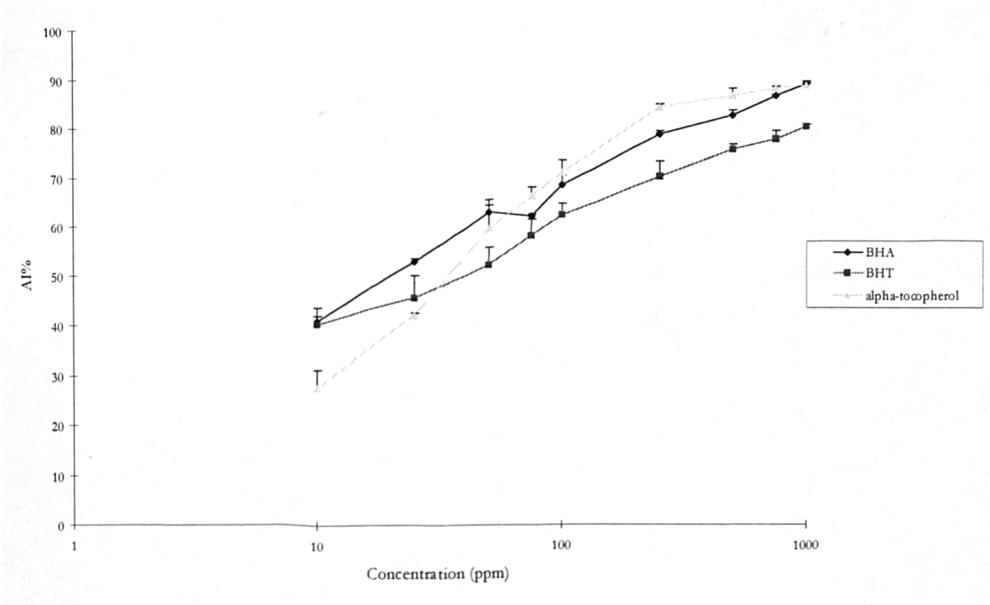


Figure 34. The antioxidant index (AI%) values for α-tocopherol and the synthetic antioxidants BHA, BHT and pyrogallol in the presence of ABAP as measured in the egg yolk TBARS assay.

5.3.2.2. Antioxidant Activities of Geranium, Melissa, Monarda, Nutmeg, Oregano and Thyme Volatile Oils in Egg Yolk, Chick Liver and Chicken Muscle Assays

The ability of the volatile oils of geranium, melissa, monarda, nutmeg, oregano and thyme to inhibit the generation of thiobarbituric acid species in egg yolk, chicken liver and muscle based systems can be seen in Figure 35 to Figure 37.

Irrespective of the lipid system used, the volatile oils demonstrated antioxidant capacities over the concentration range tested. They were able to retard the generation of malondialdehyde produced as a product of the oxidative degradation of fatty acids in each biological matrix at extreme levels of dilution. In the chicken liver system the dilution of the oils was reflected by a reduction in the percentage antioxidant index value. In general terms, the dose response for the chicken liver assay system was significantly lower than that for the egg yolk and chicken muscle systems. The egg yolk and muscle assay based systems demonstrated similar dose responses to dilutions of the oils.

Differences between the three avian models of the synthetic and volatile oils with respect to reflecting antioxidant capacities of the oils were evident. A difference in both qualitative and quantitative aspects of the lipids present within the media explains the variable responses displayed by the assays. Previous analyses of the

major lipid components and their fatty acid moieties of the tissues used in the assays were able to suggest reasons for the differential responses observed (Noble, 1987; Noble and Cocchi, 1990).

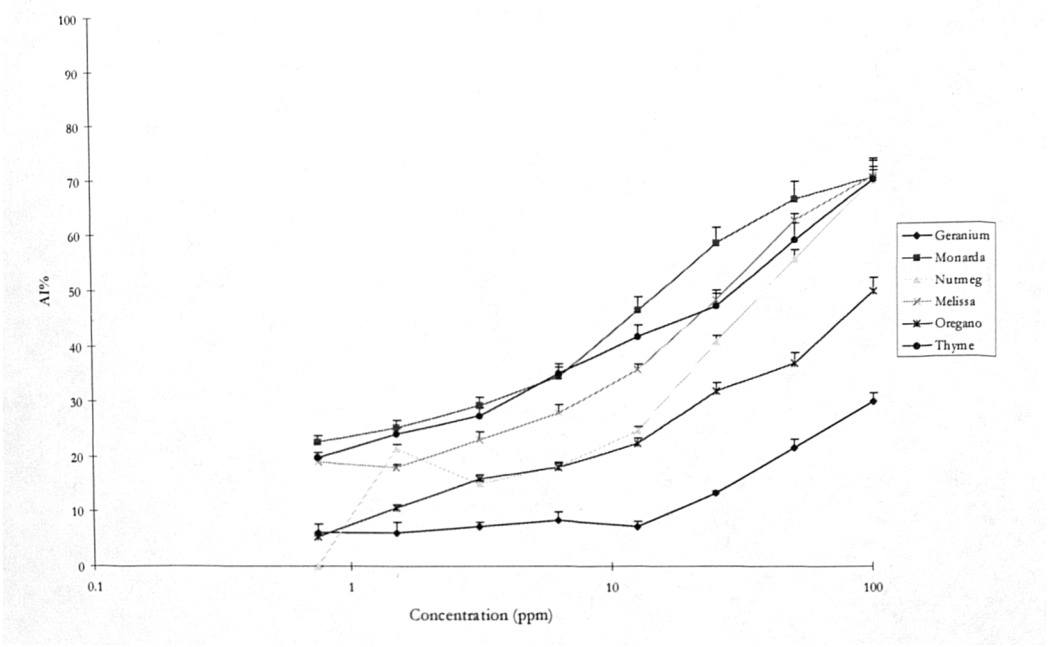


Figure 35. The antioxidant index (AI%) values for geranium, melissa, monarda, nutmeg, oregano and thyme volatile oils as measured in the egg yolk based TBARS assay.

The mean composition of the major lipids in the muscle, obtained from 30-day-old birds from a commercial source, was found to be 51% triacylglycerides, 43% phospholipids, 4% free cholesterol and 1% cholesteryl ester. The average lipid composition of the egg yolk was found to be 63% triacylglycerides, 28% phospholipids, 5% free cholesterol and 1% cholesteryl ester. The chick liver tissue contained 5% triacylglycerides, 19% phospholipids, 7% free cholesterol and 62% cholesteryl ester.

Thus, although chicken livers provide a rich source with its phospholipid components being highly unsaturated, its major component comprised of cholesterol oleate. Although chick muscle provides a substrate with higher overall levels of PUFAs, the egg yolk is comprised solely of triacylglycerides and phospholipids, both of which are highly unsaturated and therefore make the egg yolk very susceptible to lipid peroxidation, particularly to the generation of malondialdehyde which requires a minimum presence of three methylene interrupted unsaturated carbon to carbon double bonds (Pryor *et al.*, 1993).

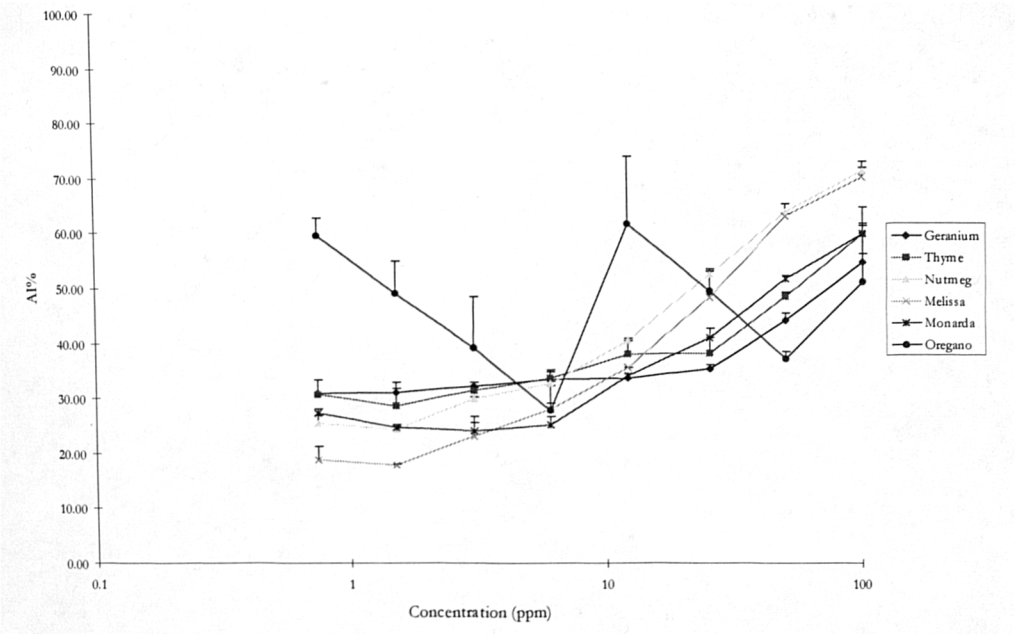


Figure 36. The antioxidant index (AI%) values for geranium, melissa, monarda, nutmeg, oregano and thyme volatile oils as measured in the liver based TBARS assay.

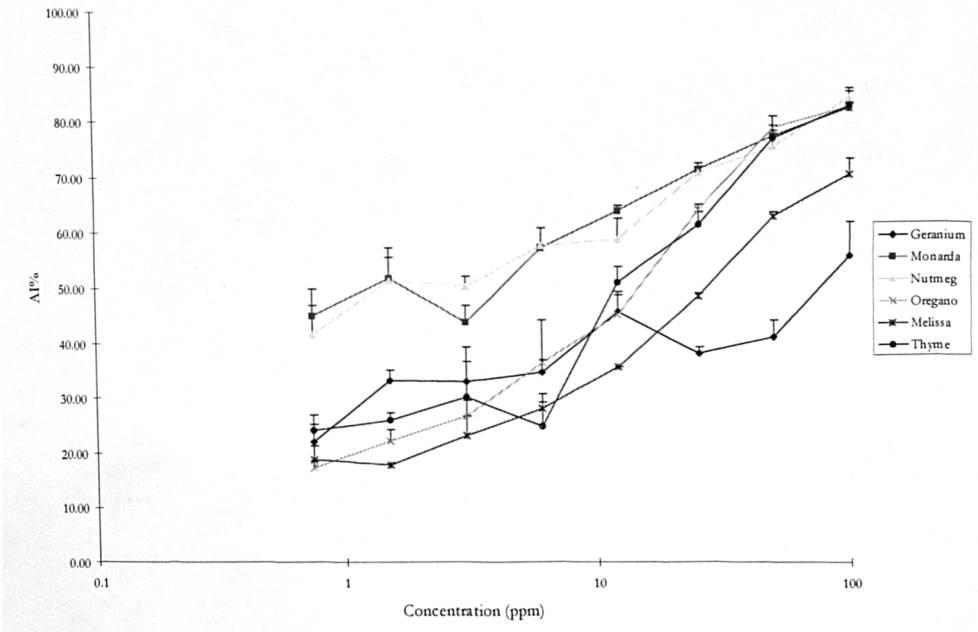


Figure 37. The antioxidant index (AI%) values for geranium, melissa, monarda, nutmeg, oregano and thyme volatile oils as measured in the muscle based TBARS assay.

In the presence of ABAP and $FeSO_4$, all the volatile oils demonstrated some degree of antioxidant activity across the concentration range utilized in the egg yolk medium (Figure 38 and Figure 39). The maximum inhibition was demonstrated to be

BHT with an Antioxidant Index % value 93.55±0.32 at 1000ppm, relative to 87.20±0.39 and 63.19±0.88% for BHA and α-tocopherol respectively. In terms of averaged activity, however, BHA and α-tocopherol demonstrated a greater degree of protection over the total concentration range than BHT, which had the greatest rate of change.

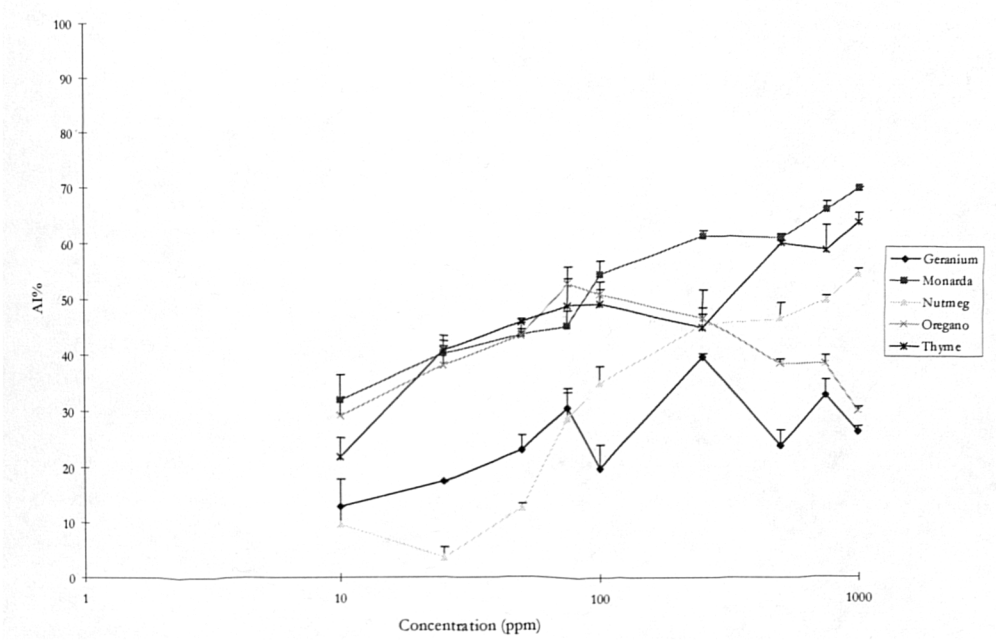


Figure 38. Antioxidant index (AI%) values for geranium, nutmeg, monarda, oregano and thyme in the presence of the water soluble azo polymerisation initiator APAB as measured in the egg yolk TBARS assay.

The ability to retard peroxidative changes in a lipid rich system of geranium, nutmeg, monarda, oregano and thyme plant volatile oils were using egg yolk as the lipid rich media and 1mM $FeSO_4$ as the inducer of lipid peroxidation is shown in Figure 39.

Geranium over this concentration range was the least effective with a very flat profile. Excepting the AI% of 18.22±2.74 at 1000ppm, the activity was constant between the concentrations 750ppm (AI% 30.50±2.22) through to 4ppm (AI% 21.27±2.90). From this data it is not possible to describe this oil as possessing limited antioxidant activity as the data would be more conclusive over a wider concentration range, as this graph may only be showing the first phase, i.e. the non-activity plateau of a classical dose-response sigmoidal curve. Nutmeg appeared to have significant antioxidant activity with AI% of 71.16±1.06 at 1000ppm, decreasing through to

7.81±0.06 at 4ppm. Compared to the activity exhibited by BHA, it was less effective at every activity: concentration point at $P<0.001$, however. This was also a characteristic of this oil relative to the other synthetic antioxidant BHT. This trend changed when compared to α-tocopherol.

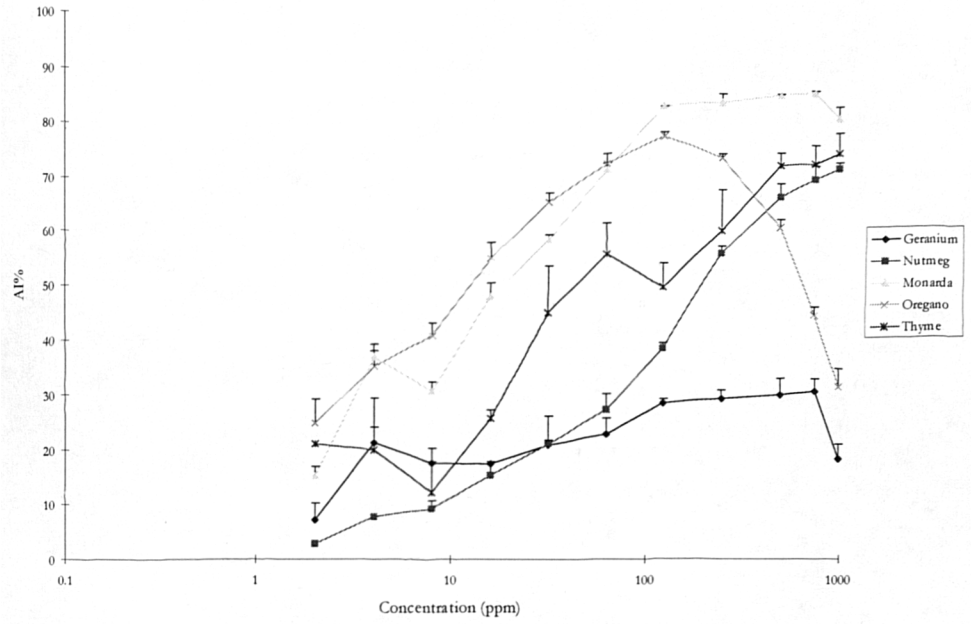


Figure 39. The antioxidant index (AI%) values for geranium, nutmeg, monarda, oregano and thyme in the presence of 1mM $FeSO_4$ as measured in the egg yolk TBARS assay.

Nutmeg was more effective at preventing the generation of MDA-$(TBA)_2$ complex thiobarbituric acid reactive species at 1000ppm ($P<0.01$) and 750ppm ($P<0.05$) and demonstrated similar activities at concentrations 500ppm and 250ppm.

Monarda demonstrated very high antioxidant activities in this assay system, ranging from 80.42±2.09 at 1000ppm through to an AI% value of 15.24±1.67 at 2ppm. The oil was statistically similar in action to that of BHA at concentrations 1000 through 250ppm and at 125ppm. BHT was more effective throughout the concentration range. Compared to α-tocopherol, monarda was statistically better or was of similar potency at inhibiting lipid peroxidation. Only at the last concentration point was monarda significantly different. The profile of the oil from oregano was

uniquely characteristic when compared to BHA, BHT, α-tocopherol and the other oils.

Oregano generated a bell shaped curvature, with a maximum AI% of 76.99±0.85 at 125ppm, starting from an AI% of 31.33±3.31 at 1000ppm and an AI% of 24.81±4.45 at the lowest concentration tested. Inspite of repeated vigorous control of the experimental procedures, no suitable explanation could be found. When compared to BHA and BHT, oregano was less effective at preventing peroxidation of the lipid rich egg yolk. Thyme oil was highly effective at inhibiting TBARS over the concentration range tested. With the exception of the oil at 8ppm, the antioxidant capacity decreased fairly constantly over the range from an AI% of 74.04±3.44 at 1000ppm to an AI% 21.02±2.59 at the lowest concentration. Comparisons with BHA and BHT showed that thyme oil was less effective than these synthetic compounds. Thyme was statistically less effective at concentrations 1000, 500 and 250ppm ($P<0.05$) while at 750 ppm, 125 ppm, 63 ppm, 32 ppm and 4ppm ($P<0.01$) and at 16 ppm and 8ppm ($P<0.001$) than BHA.

In the case of α-tocopherol, thyme oil was more effective than α-tocopherol at concentrations 1000ppm through 500ppm ($P<0.01$). At concentrations 250ppm through 32ppm, thyme was statistically insignificant from the activity expressed by this compound.

The order of activity across the concentration ranges used was found to be monarda > thyme > nutmeg > geranium. When their activities were compared to that of the synthetic antioxidant BHA, the activity of the volatile oils were found to be significantly different on an activity: concentration basis, being less effective at inhibiting the generation of TBARS. The exception was found to be monarda, which at concentration points 250 ppm and 125ppm were found to be statistically insignificantly different to the activity for the corresponding BHA data points as shown in Table 38.

The antioxidant activities of BHA, BHT and α-tocopherol were assessed by the modified TBARS assay in the presence of the water soluble azo polymerisation initiator 2,2-azobis (2-amidinopropane) dihydrochloride (ABAP), Figure 34. The activities of geranium, nutmeg, monarda, oregano and thyme oils were also assessed under the same experimental conditions, Figure 38. The activity of BHA, BHT and

α-tocopherol were very similar when tested with this inducer of lipid peroxidation. α-tocopherol was less active at the two lowest concentrations. The activities of BHA, BHT and α-tocopherol were very similar when tested with this inducer of lipid peroxidation. α-tocopherol was less active at the two lowest concentrations.

Geranium was the least active and the most unstable oil tested, increasing in activity from 10ppm (AI%: 12.86±0.00) through to 250ppm (AI%: 40.26±0.52) after which it tended to moderate downwards, AI%: 26.61±0.89 at 1000ppm. Relative to BHA, BHT and α-tocopherol, it was significantly less effective under these experimental conditions.

Monarda demonstrated the greatest antioxidant activity of the tested volatile oils, having an AI%: 71.03±0.20 at 1000ppm as compared to thyme (AI%: 64.70±1.79), nutmeg (AI%: 55.23±1.03), oregano (AI%: 30.44±0.66) and geranium (AI%: 26.61±0.89). Relative to BHA, monarda was significantly less active throughout the concentration range. This was less obvious in the case of BHT and α-tocopherol especially at lower concentrations.

Nutmeg demonstrated significant antioxidant ability: AI% 71.16 at 1000ppm, decreasing to an AI% value of 7.81% at 4ppm. Compared to the activity of BHA, it was less effective at each data point ($P<0.001$). This was also a characteristic of the oil against the other synthetic antioxidant BHT. This trend was not the case against α-tocopherol. Nutmeg was significantly more effective at 1000ppm ($P<0.01$) and at concentration point 750ppm, though less so ($P<0.05$). At concentrations 500ppm and 250ppm it demonstrated similar activity.

Oregano demonstrated a bell shaped profile, reaching a maximum AI% at 125ppm of 76.99±0.85, starting from an AI% 31.33±3.31 at 1000ppm and an AI% 24.81% at the lowest concentration tested. The reason for this characteristic profile is not clear. In comparisons with the activity of BHA and BHT, oregano was significantly less effective at preventing thiobarbituric acid reactive species being generated. Similar comparisons with α-tocopherol revealed that this oil was significantly less effective statistically at concentrations, 1000pppm and 750ppm.

Thyme was very effective at inhibiting peroxidation, only being less active than monarda at high concentrations and at the lowest. Its action was significantly

less active than BHA and BHT at this concentration range but less so when compared to α-tocopherol.

Table 38. The antioxidant index (AI%) values for geranium, nutmeg, monarda, oregano and thyme in the presence of 1mM $FeSO_4$ relative to the activity of the synthetic antioxidant BHA as measured in the egg yolk TBARS assay.

Concentration (ppm)	BHA	Geranium	Nutmeg	Monarda	Oregano	Thyme
1000	87.20±0.39	18.22±2.74^{c}	71.16±1.0^{c}	80.42±2.09^{a}	31.33±3.3^{c}	74.04±3.44^{a}
750	86.56±0.79	30.50±2.2^{c}	68.96±2.4^{c}	84.75±0.43^{a}	44.08±1.6^{c}	71.83±3.39^{b}
500	86.77±0.42	29.89±3.0^{c}	65.82±2.3^{c}	84.41±0.15^{b}	60.08±1.4^{c}	71.67±2.40^{a}
250	82.83±0.62	29.00±1.5^{c}	55.26±1.4^{c}	83.04±1.63	72.99±0.7^{c}	59.44±7.60^{a}
125	81.42±0.68	28.27±0.7^{c}	38.24±0.9^{c}	82.50±0.07	76.99±0.85^{b}	49.31±4.26^{b}
63	80.81±1.49	22.83±2.7^{c}	27.24±2.7^{c}	70.83±1.37^{b}	71.96±2.03^{a}	55.30±5.66^{b}
32	83.89±1.07	20.82±1.10^{Ic}	15.95±2.30^{Ic}	58.14±1.0^{c}	65.24±1.6^{c}	44.79±8.66^{b}
16	81.97±0.84	17.48±0.33^{Ic}	15.37±2.50^{Ic}	48.02±2.6^{c}	55.17±2.7^{c}	25.81±1.60^{Ic}
8	77.04±1.11	17.67±2.7^{c}	9.28±1.50^{Ic}	30.88±1.6^{c}	40.90±2.3^{c}	12.35±5.50^{Ic}
4	70.38±2.04	21.27±2.90^{Ic}	7.81±0.06^{Ic}	36.89±2.1^{c}	35.08±2.9^{c}	19.90±9.50^{b}
2	62.59±0.73	7.16±3.1^{c}	2.83±^{II}	15.24±1.6^{c}	24.81±4.4^{c}	21.02±^{II}

All values are expressed as the mean±S.E.M. of three replicates, except ^{I}: mean of two replicates and ^{II}: one replicate. Statistical analysis was carried out using a Student t-test using a pooled standard deviation. ^{a} $P<0.05$ ^{b} $P<0.01$ ^{c} $P<0.001$.

Table 39. The antioxidant index (AI%) values for geranium, nutmeg, monarda, oregano and thyme in the presence of 1mM $FeSO_4$ relative to the activity of the synthetic antioxidant BHT as measured in the egg yolk TBARS assay.

Concentration (ppm)	BHT	Geranium	Nutmeg	Monarda	Oregano	Thyme
1000	93.55±0.32	18.22±2.74^{c}	71.16±1.06^{c}	80.42±2.09^{b}	31.33±3.31^{c}	74.04±3.44^{b}
750	93.43±0.16	30.50±2.22^{c}	68.96±2.44^{c}	84.75±0.43^{Ic}	44.08±1.61^{c}	71.83±3.39^{b}
500	92.67±0.52^{I}	29.89±3.08^{c}	65.82±2.37^{b}	84.41±0.15^{c}	60.08±1.45^{c}	71.67±2.40^{b}
250	90.33±0.51	29.00±1.56^{c}	55.26±1.42^{c}	83.04±1.63^{b}	72.99±0.75^{c}	59.44±7.60^{b}
125	87.83±0.77	28.27±0.76^{c}	38.24±0.99^{c}	82.50±0.07^{b}	76.99±0.85^{c}	49.31±4.26^{c}
63	83.27±1.01	22.83±2.79^{c}	27.24±2.75^{c}	70.83±1.37^{b}	71.96±2.03^{b}	55.30±5.66^{b}
32	78.77±0.50	20.82±1.10^{Ic}	15.95±2.30^{Ic}	58.14±1.08^{c}	65.24±1.63^{b}	44.79±8.66^{b}
16	68.93±1.94	17.48±0.33^{Ic}	15.37±2.50^{Ic}	48.02±2.64^{b}	55.17±2.79^{b}	25.81±1.60^{Ic}
8	54.48±4.05	17.67±2.71^{b}	9.28±1.50^{Ib}	30.88±1.62^{b}	40.90±2.33^{c}	12.35±5.50^{Ib}
4	44.39±0.59	21.27±2.90^{Ib}	7.81±0.06^{Ic}	36.89±2.18^{b}	35.08±2.91^{b}	19.90±9.50^{a}
2	38.29±3.20	7.16±3.15^{b}	2.83±^{II}	15.24±1.67^{c}	24.81±4.45^{b}	21.02±^{II}

All values are expressed as the mean±S.E.M. of three replicates, except ^{I}: mean of two replicates and ^{II}: one replicate. Statistical analysis was carried out using a Student t-test using a pooled standard deviation. ^{a} $P<0.05$ ^{b} $P<0.01$ ^{c} $P<0.001$.

Table 40. The antioxidant index (AI%) values for geranium, nutmeg, monarda, oregano and thyme in the presence of 1mM $FeSO_4$ relative to the activity of α-tocopherol as measured in the egg yolk TBARS assay.

Concentration (ppm)	α-Tocopherol	Geranium	Nutmeg	Monarda	Oregano	Thyme
1000	63.19±0.88	18.22±2.74[b]	71.16±1.06[b]	80.42±2.09[b]	31.33±3.31[c]	74.04±3.44[a]
750	62.22±1.87	30.50±2.22[b]	68.96±2.44[a]	84.75±0.43[Ib]	44.08±1.61[b]	71.83±3.39[a]
500	57.77±2.76	29.89±3.08[b]	65.82±2.37	84.41±0.15[c]	60.08±1.45	71.67±2.40[a]
250	55.76±2.76	29.00±1.56[b]	55.26±1.42	83.04±1.63[c]	72.99±0.75[b]	59.44±7.60
125	55.52±1.92	28.27±0.76[c]	38.24±0.99[c]	82.50±0.07[c]	76.99±0.85[c]	49.31±4.26
63	53.79±2.52	22.83±2.79[c]	27.24±2.75[b]	70.83±1.37[b]	71.96±2.03[b]	55.30±5.66
32	52.10±4.15	20.82±1.10[Ib]	21.07±5.2[b]	58.14±1.08	65.24±1.63[a]	44.79±8.66
16	50.96±2.16	17.48±0.33[b]	15.37±2.50[Ib]	48.02±2.64	55.17±2.79	25.81±1.60[Ib]
8	43.34±5.84[I]	17.67±2.71[a]	9.28±1.50[Ia]	30.88±1.62	40.90±2.33	12.35±5.50[Ib]
4	41.92±2.91	21.27±2.90[Ib]	7.81±0.06[Ib]	36.89±2.18	35.08±2.91	19.90±9.50[a]
2	34.16±1.51	7.16±3.15[c]	2.83±[II]	15.24±1.67[c]	24.81±4.45	21.02±[II]

All values are expressed as the mean±S.E.M. of three replicates, except [I]: mean of two replicates and [II]: one replicate. Statistical analysis was carried out using a Student t-test using a pooled standard deviation. [a] $P<0.05$ [b] $P<0.01$ [c] $P<0.001$.

Table 41. The antioxidant index (AI%) values for five volatile oils at 9 concentrations (1000ppm to 10ppm) as measured in the egg yolk TBARS assay, in the presence of the water soluble azo polymerisation initiator ABAP.

Concentration (ppm)	BHA	Geranium	Indonesian Nutmeg	Monarda	Oregano	Thyme
1000	88.71±0.63	26.61±0.89^{c}	55.23±1.03^{c}	71.03±0.20^{c}	30.44±0.66^{c}	64.70±1.79^{c}
750	86.43±0.38	33.18±2.88^{c}	50.51±0.65^{c}	67.02±1.45^{c}	38.81±1.67^{lc}	59.60±4.59^{b}
500	82.52±0.97	24.12±2.82^{c}	47.16±3.01^{c}	62.01±0.84^{c}	39.12±0.78^{c}	61.08±0.48^{lc}
250	78.64±0.68	40.26±0.52^{c}	45.90±3.16^{c}	62.19±1.04^{c}	47.18±0.89^{c}	45.56±6.79^{b}
100	68.28±1.89	19.93±4.25^{c}	35.38±2.87^{c}	54.83±2.43^{b}	51.24±0.95^{c}	49.59±3.84^{b}
75	61.68±0.46	30.84±3.01^{c}	29.01±5.58^{b}	45.70±2.68^{b}	53.37±0.97^{c}	49.36±6.95^{lb}
50	62.54±1.51	23.24±2.62^{lb}	12.80±0.71^{c}	43.93±1.09^{c}	43.84±2.33^{b}	46.36±4.40^{lc}
25	52.32±0.82	17.49±^{ll}	3.58±1.88^{lc}	40.34±1.35^{c}	38.41±4.19^{b}	41.00±2.60^{lb}
10	40.95±1.12	12.86±0.00^{lb}	9.67±8.12^{lc}	32.16±4.48^{a}	29.17±3.41^{b}	21.88±3.34^{b}

N.B. Values are expressed as the mean ±S.E.M. of three replicates except ^{a} mean of two replicates and ^{b} only one replicate. Statistical analysis was carried out using a Student t-test using a pooled standard deviation. * $P<0.05$ ** $P<0.01$ *** $P<0.001$.

Table 42. The antioxidant index (AI%) values for five volatile oils at 9 concentrations (1000ppm to 10ppm) as measured in the egg yolk TBARS assay, in the presence of the water soluble azo polymerisation initiator ABAP.

Concentration (ppm)	BHT	Geranium	Indonesian Nutmeg	Monarda	Oregano	Thyme
1000	80.21±0.46	26.61±0.89^{c}	55.23±1.03^{c}	71.03±0.20^{b}	30.44±0.66^{c}	64.70±1.79^{b}
750	77.67±1.76	33.18±2.88^{c}	50.51±0.65^{c}	67.02±1.45^{b}	38.81±1.67^{Ic}	59.60±4.59^{b}
500	75.61±1.06	24.12±2.82^{c}	47.16±3.01^{c}	62.01±0.84^{c}	39.12±0.78^{c}	61.08±0.48^{Ic}
250	70.16±3.01	40.26±0.52^{b}	45.90±3.16^{c}	62.19±1.04	47.18±0.89^{c}	45.56±6.79^{a}
100	62.19±2.24	19.93±4.25^{c}	35.38±2.87^{c}	54.83±2.43^{a}	51.24±0.95^{b}	49.59±3.84^{a}
75	57.75±3.38	30.84±3.01^{b}	29.01±5.58^{b}	45.70±2.68^{a}	53.37±0.97^{Ic}	49.36±6.95^{I}
50	51.80±3.60	23.24±2.62^{Ib}	12.80±0.71^{c}	43.93±1.09^{a}	43.84±2.33^{a}	46.36±4.4^{Ia}
25	45.35±4.31	17.49±^{II}	3.58±1.88^{c}	40.34±1.35^{a}	38.41±4.19^{a}	41.00±2.6^{Ia}
10	40.43±3.30	12.86±0.00^{Ib}	9.67±8.12^{Ib}	32.16±4.48^{a}	29.17±3.41^{a}	21.88±3.34^{b}

N.B. Values are expressed as the mean ±S.E.M. of three replicates except ^{I} mean of two replicates and ^{II} only one replicate. Statistical analysis was carried out using a Student t-test using a pooled standard deviation. ^{a} $P<0.05$ ^{b} $P<0.01$ ^{c} $P<0.001$.

Table 43. The antioxidant index (AI%) values for five volatile oils at 9 concentrations (1000ppm to 10ppm) as measured in the egg yolk TBARS assay, in the presence of the water soluble azo polymerisation initiator ABAP.

Concentration (ppm)	α-Tocopherol	Geranium	Indonesian Nutmeg	Monarda	Oregano	Thyme
1000	88.53±0.49	26.61±0.89[c]	55.23±1.03[c]	71.03±0.20[c]	30.44±0.66[c]	64.70±1.79[b]
750	87.90±0.60	33.18±2.88[c]	50.51±0.65[c]	67.02±1.45[c]	38.81±1.67[Ic]	59.60±4.59[b]
500	86.59±1.47	24.12±2.82[c]	47.16±3.01[c]	62.01±0.84[c]	39.12±0.78[c]	61.08±0.48[Ic]
250	84.16±0.58	40.26±0.52[c]	45.90±3.16[c]	62.19±1.04[c]	47.18±0.89[c]	45.56±6.79[b]
100	70.70±2.55	19.93±4.25[c]	35.38±2.87[c]	54.83±2.43[b]	51.24±0.95[b]	49.59±3.84[a]
75	65.70±1.90	30.84±3.01[c]	29.01±5.58[c]	45.70±2.68[b]	53.37±0.97[b]	49.36±6.95[I]
50	59.09±5.95	23.24±2.62[Ia]	12.80±0.71[b]	43.93±1.09[b]	43.84±2.33[a]	46.36±0.44[I]
25	41.83±0.53	17.49±[I]	3.58±1.88[Ic]	40.34±1.35	38.41±4.19	41.00±2.60[I]
10	27.65±3.42	12.86±0.00[Ib]	9.67±8.12[c]	32.16±4.48	29.17±3.41	21.88±3.34

N.B. Values are expressed as the mean ±S.E.M. of three replicates except [I] mean of two replicates and [II] only one replicate. Statistical analysis was carried out using a Student t-test using a pooled standard deviation. [a] $P<0.05$ [b] $P<0.01$ [c] $P<0.001$.

The antioxidant capacity of the volatile oils of clove and nutmeg and their phytoconstituents were assessed in a rat liver TBARS assay can be seen in Figure 40 to Figure 43. All the components tested clearly demonstrated the ability to inhibit the generation of MDA produced by the breakdown of endoperoxides resulting from the oxidation of fatty acids in a dose-dependent fashion.

5.3.2.3. Antioxidant Activities of Clove and Nutmeg Volatile Oils and Components in the Rat Liver TBARS Assays

As shown in Figure 40, both the volatile oils demonstrated some degree of antioxidant capacity in this TBARS assay. The oils demonstrated clear sigmoidal dose response curves, over the concentration range tested. The IC_{50} values were 20ppm, 100ppm for clove and nutmeg oils respectively. The plateau for nutmeg was just higher than for clove. Both, however, gradually declined in measured antioxidant capacity from the same concentration point. The slope of this decline was greatest for clove.

The antioxidant capacities of a number of the components found in these volatile oils can be seen in Figure 41 to Figure 43. As can be seen, the components all demonstrated some degree of activity in this assay system.

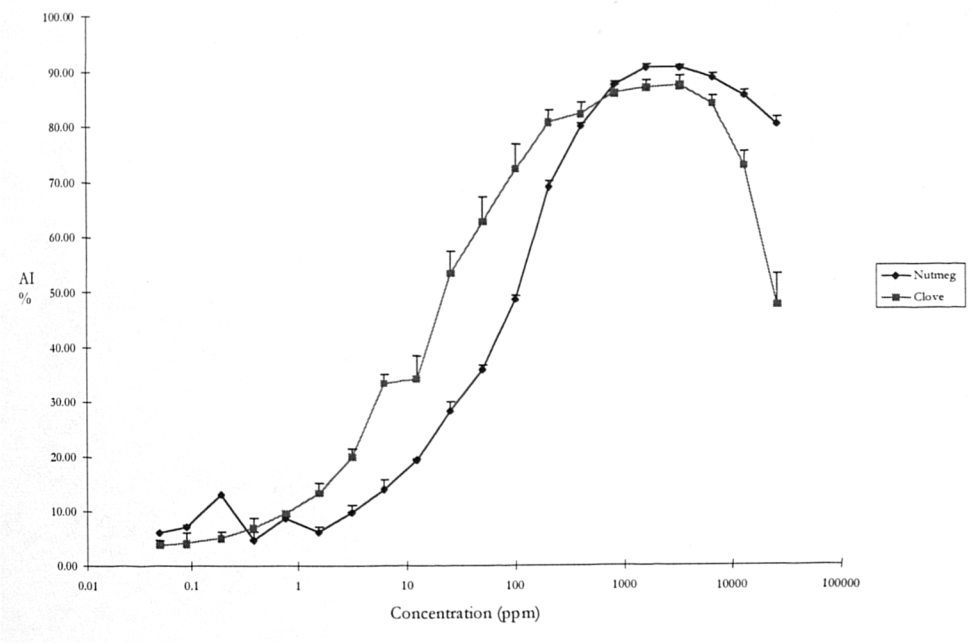


Figure 40. The antioxidant capacity of black pepper and clove volatile oils as measured by the thiobarbituric acid assay using rat liver *homogenate.*

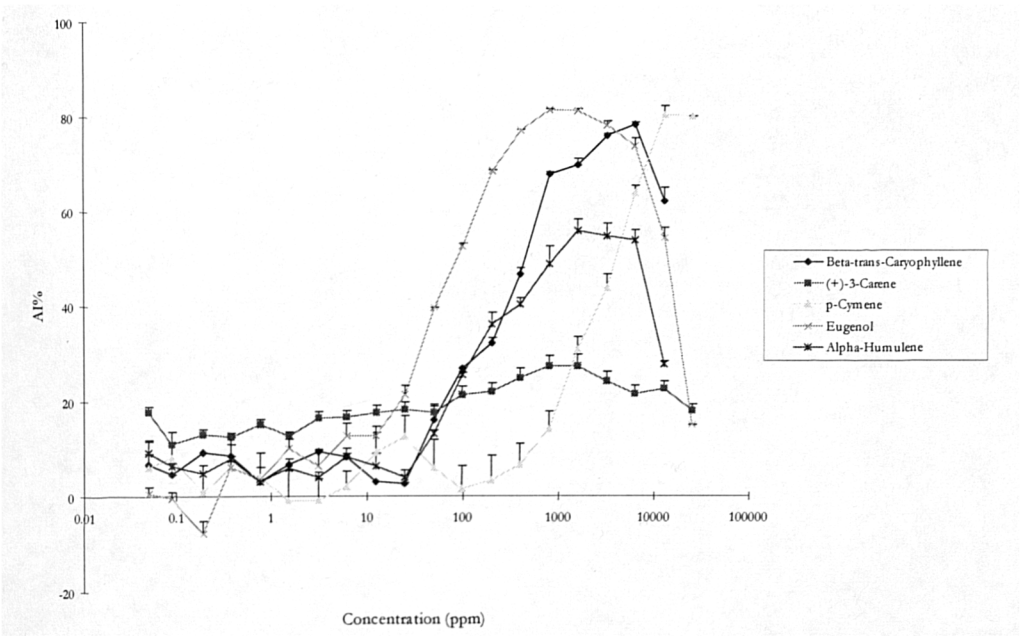


Figure 41. The antioxidant activity of β-*trans*-caryophyllene, δ-3-carene, *p*-cymene, eugenol and α-humulene as measured in rat liver TBARS assay.

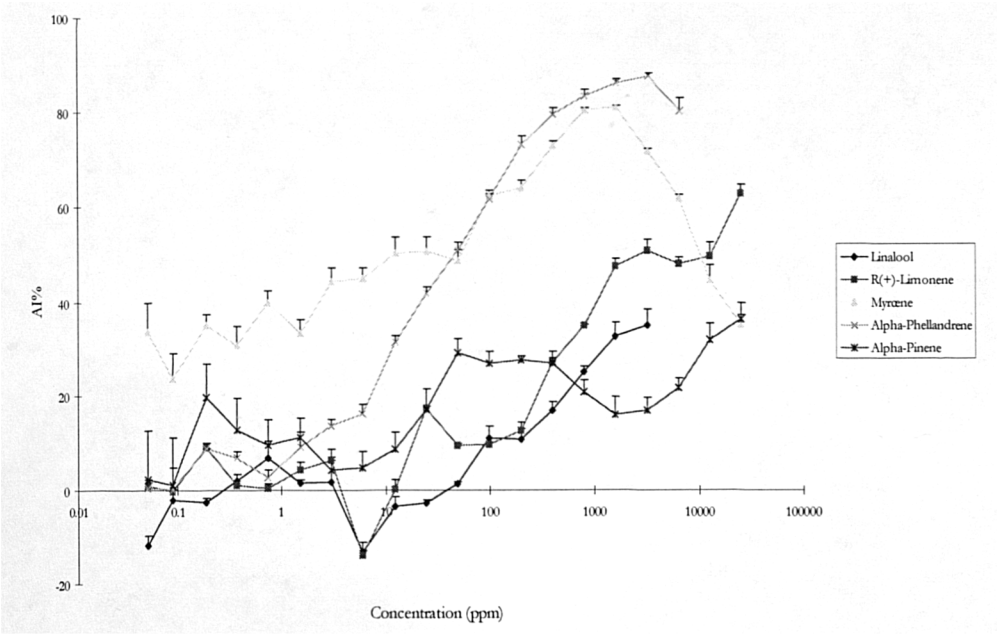


Figure 42. The antioxidant activity of linalool, R (+)-limonene, myrcene, α-phellandrene and α-pinene as measured in rat liver TBARS assay.

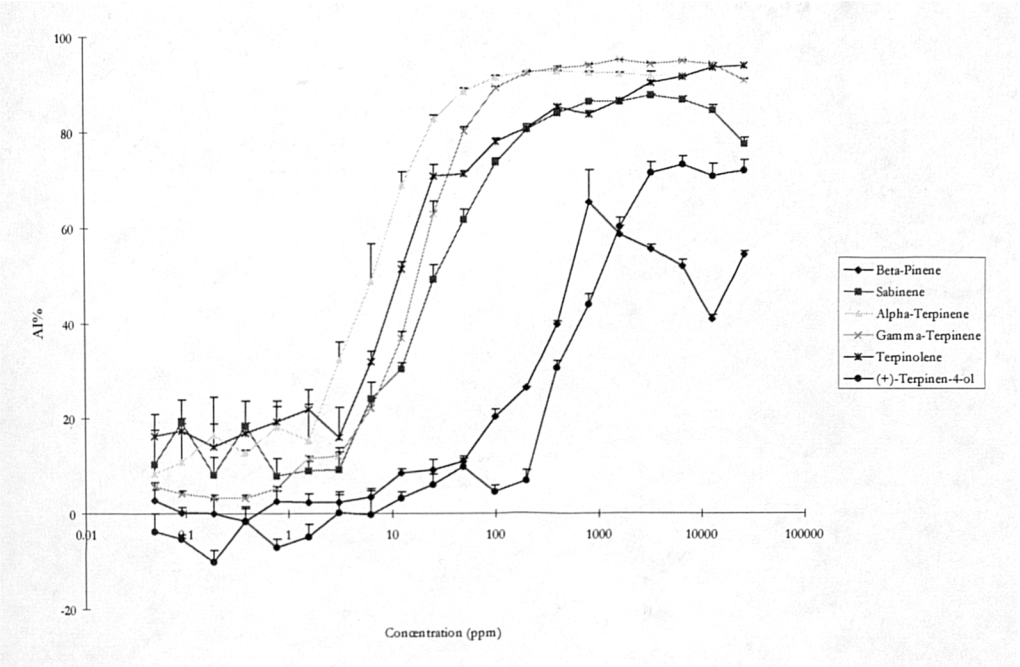


Figure 43. The antioxidant activity of β-pinene, sabinene, α-terpinene, γ-terpinene, terpinolene and (+)-terpinen-4-ol as measured in rat liver TBARS assay.

5.3.3. Spectrophotometric Conjugated Diene Assay

The relative antioxidant efficiencies of a variety of compounds with antioxidant properties can be seen in Table 44. The order of activity was found to be Trolox > (+)-α-tocopherol > (+)-δ-tocopherol > pyrogallol > BHA > BHT > β-carotene.

Table 44. The relative antioxidant efficiency of known antioxidants [2.5mM].

Compound	RAE
(+)-α-Tocopherol	1.000
(+)-δ-Tocopherol	0.910
β-Carotene	0.137
BHA	0.797
BHT	0.795
Pyrogallol	0.886
Trolox	1.059

Each value is expressed as the mean of three independent experiments.

The relative antioxidant efficiency for the volatile oils black pepper, clove, clove bud, clove terpenes, geranium, lovage leaf, lovage stem, melissa, monarda, nutmeg, oregano, different pimento oils and thyme are shown in Table 45. The order of activity was found to be lovage leaf > clove > clove bud > pimento[2] > oregano > black pepper > nutmeg > clove terpenes > lovage stem and geranium.

The relative antioxidant efficiency of a number of the components found in the volatile oils from Table 45 are shown in Table 46. The order of activity was found to be eugenol > thymol > carvacrol > terpinolene > 1,8-cineole > nerol > (+)-terpinen-4-ol > carvacrol methyl ether > α-terpineol > menthone > citral (*cis*+*trans*) > geranyl acetate > geraniol > linalool > myrcene > *p*-cymene > citronellal.

In SDS micelles, ABAP partitions approximately 91% into the micellar phase (Pryor *et al.,* 1993) and has a distribution similar to the 95% reported for linoleic acid. Therefore, one would expect the majority of the initial radicals generated from the thermolytic decomposition of ABAP to be produced either within or at the surface of the micelle. Lipid soluble inhibitors would be expected to act by

scavenging lipid peroxyl radicals while water soluble inhibitors would be expected to react with either the small percentage of radicals generated in the water phase from ABAP or at the surface of the micelle, reacting with the primary radicals and the more numerous lipid peroxyl radicals (Pryor *et al.*, 1993).

Table 45. The relative antioxidant efficiency of various volatile oils at 1000ppm.

Compound	RAE
Black pepper	0.260
Clove[1]	0.481
Clove bud oil[2]	0.445
Clove terpenes[2]	0.155
Geranium[3]	0.091
Lovage (Leaf)	0.840
Lovage (Stem)	0.128
Nutmeg	0.251
Oregano	0.361
Pimento[2]	0.437

Each value is expressed as the mean of three independent experiments. **1**: Butterworth and Sage; **2**: R.C. Treatt; **3**: Meadows Canterbury Ltd.

Table 46. The relative antioxidant efficiency of the major components from the tested volatile oils [2.5mM].

Component	RAE
Carvacrol	0.362
Carvacrol, methyl ether	0.122
1,8-Cineole	0.157
Citral (*cis+trans*)	0.112
Citronellal	0.099
p-Cymene	0.105
Eugenol	0.471
Geraniol	0.111
Geranyl acetate	0.112
Linalool	0.109
Menthone	0.113
Myrcene	0.106
Nerol	0.141
(+)-Terpinen-4-ol	0.123
α-Terpineol	0.117
Terpinolene	0.228
Thymol	0.368

Each value is expressed as the mean of three independent experiments.

5.3.4. Total Antioxidant Activity (Radical TRAP) Assay

The free radical-trapping activities of the natural antioxidant α-tocopherol at 2.5mM; the synthetic antioxidants BHA, BHT and pyrogallol at 2.5mM relative to the water soluble α-tocopherol analogue Trolox (2.5mM) are shown in Table 47. The free radical-trapping activities of BHA, BHT and pyrogallol at 0.05mM to 2.5mM concentration levels and α-tocopherol at 0.20mM to 2.5mM concentration levels are shown in Figure 45 and Figure 46. The order of activity was determined as BHA > pyrogallol > BHT > α-tocopherol. The most effective antioxidants were the synthetic antioxidants BHA and pyrogallol that demonstrated similar molar equivalent antioxidant capacities at 68.69±0.06mmol L^{-1} and 68.27±0.16mmol L^{-1} respectively. The determined capacities of BHT and α-tocopherol demonstrated approximately 50% less effective free radical-trapping activities than the most effective compounds

BHA and pyrogallol. Interestingly, BHT and α-tocopherol possessed approximately equivalent antioxidant capacities at 36.31±0.89mmol L^{-1} and 30.71±2.40mmol L^{-1} respectively.

Table 47. Total antioxidant status values for antioxidant compounds [2.5mM] relative to Trolox [2.5mM].

Total Antioxidant Status (TRAP) Assay	
Test Compound [2.5mM]	**Trolox Equivalent [mmol L^{-1}]**
BHA	68.69±0.06
BHT	36.31±0.89
Pyrogallol	68.27±0.16
α-Tocopherol	30.71±2.40

Each value is the mean±SEM of three independent experiments.

The free radical-trapping activities of the volatile oils of black pepper, clove, geranium, melissa, nutmeg, oregano and thyme, relative to the water soluble α-tocopherol analogue Trolox (2.5mM), are shown in Table 48. The order of activity was determined as clove > oregano > thyme > black pepper > nutmeg [Serva] > geranium > nutmeg > melissa. The free radical-trapping activities of clove and oregano volatile oils were greater at 69.05±0.06mmol L^{-1} and 61.55±0.57mmol L^{-1} respectively than the next most active volatile oil [thyme - 43.39±0.74 mmol L^{-1}] and demonstrated free radical-trapping capacity of the same magnitude as the most active antioxidants BHA and pyrogallol. Thyme volatile oil demonstrated approximately the same order of activity as the synthetic antioxidant BHT and the natural antioxidant α-tocopherol, in this assay system.

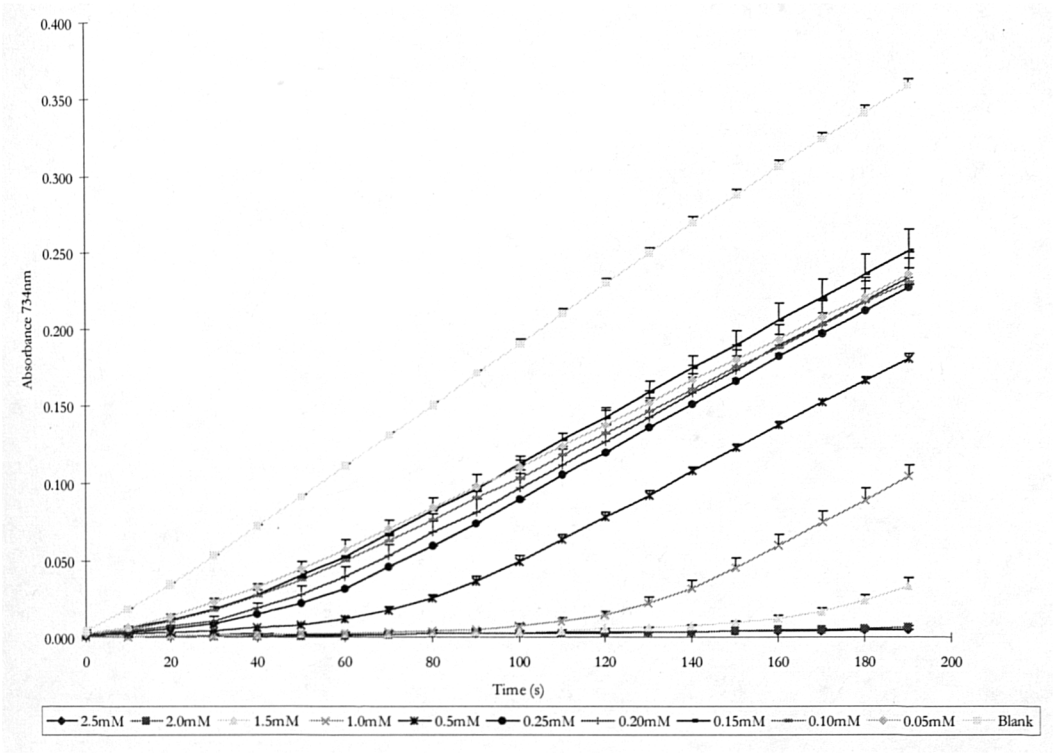
Table 48. Total antioxidant status values for volatile oils at 1000ppm relative to Trolox (2.5mM).

Total Antioxidant Status (TRAP) Assay	
Volatile Oils [1000ppm]	**Trolox Equivalent [mmol L^{-1}]**
Black Pepper	25.36±1.97
Clove	69.05±0.06
Geranium	5.84±0.39
Melissa	0.28±0.03
Nutmeg	5.00±2.14
Nutmeg [Serva]	9.76±2.42
Oregano	61.55±0.57
Thyme	43.39±0.74

Each value is the mean±SEM of three independent experiments.

The free radical-trapping activities of commercially available components determined to be present in the volatile oils used in this study, relative to the water soluble α-tocopherol analogue Trolox (2.5mM), are shown in Table 49.

(a)



(b)

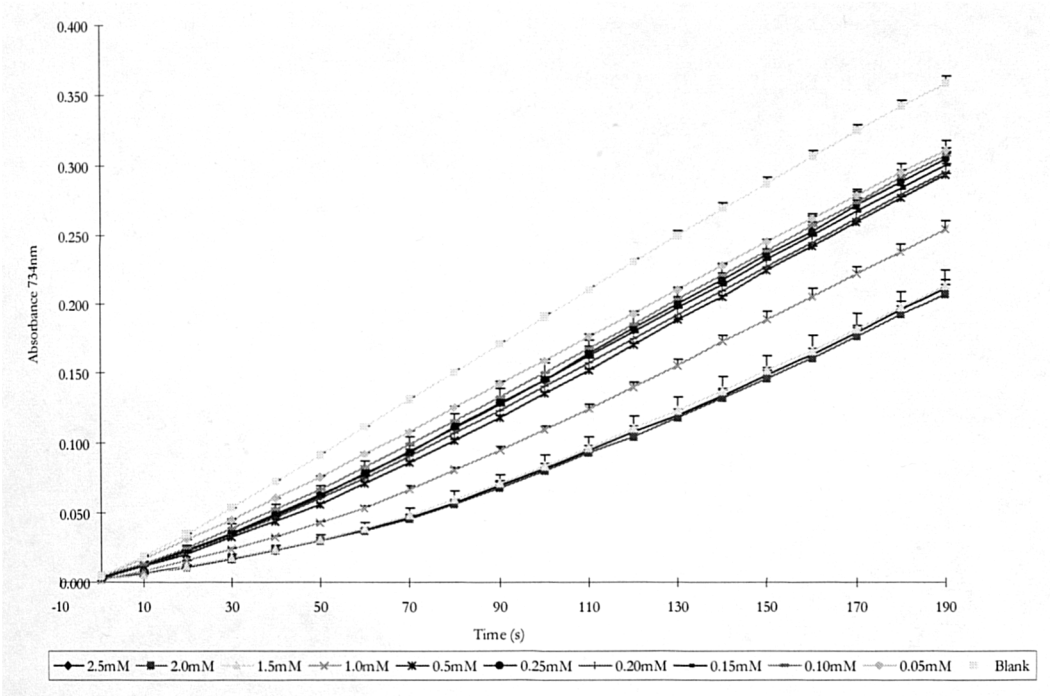
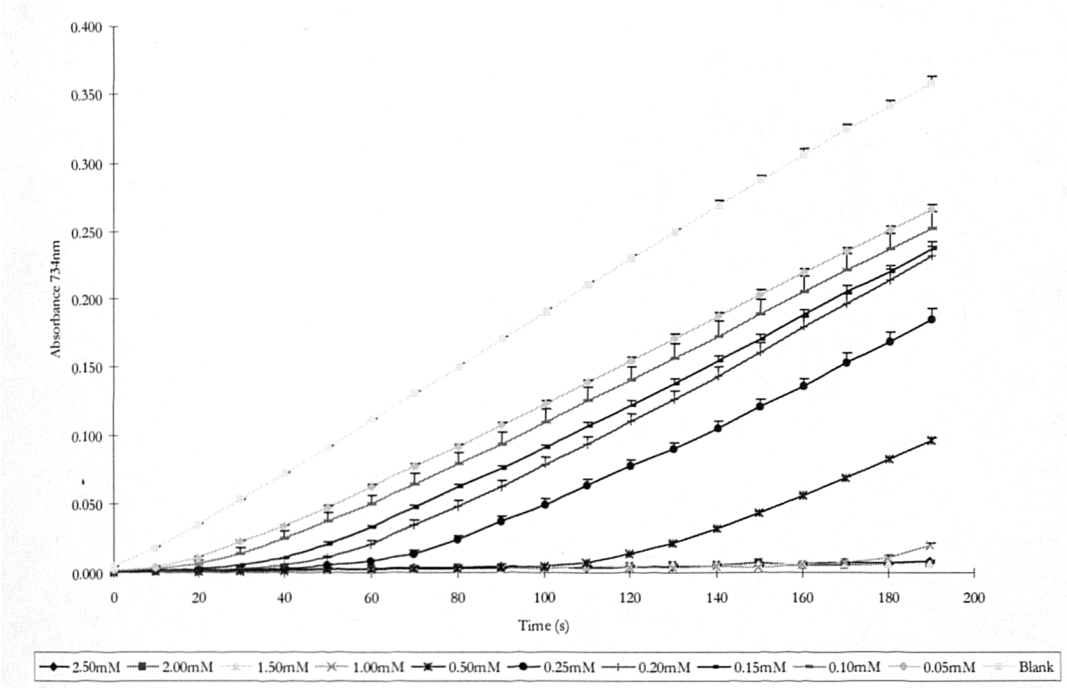


Figure 44. Free radical-trapping activity of (a) BHA and (b) BHT at 0.05mM to 2.5mM concentration levels.

(a)



(b)

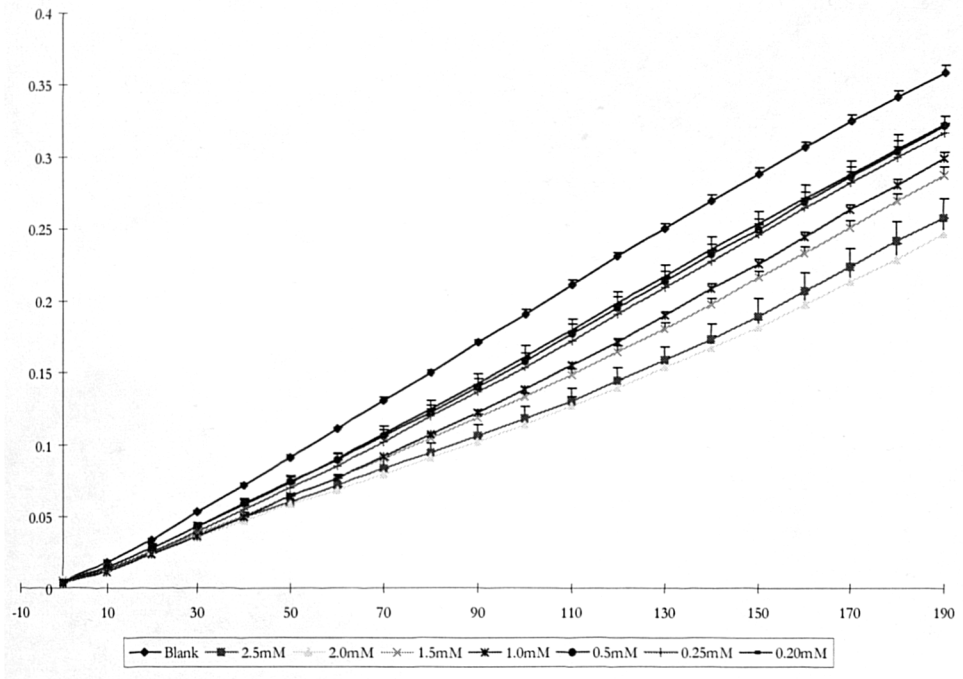


Figure 45. Free radical-trapping activity of (a) Pyrogallol at 0.05mM to 2.5mM Concentration levels and (b) α-Tocopherol at 2.5mM to 0.20mM concentration levels.

Table 49. Total antioxidant status values for phytoconstituents at 1000ppm relative to Trolox [2.5mM].

Total Antioxidant Status (TRAP) Assay	
Phytoconstituent [1000ppm]	**Trolox Equivalent [mmol L^{-1}]**
Aromadendrene	0.74±0.16
α-Bisabolol	5.18±0.78
Borneol	18.66±3.13
trans-β-Caryophyllene	7.77±0.98
δ-3- Carene	3.93±0.63
1,8-Cineole	1.13±0.70
Carvacrol	58.93±2.79
Carvacrol methyl ether	15.00±1.13
Cedrene	21.61±0.93
cis-3-Hexan-1-ol	18.51±1.21
Citral (*cis*+*trans*)	13.45±0.86
Citronellal	22.50±5.67
p-Cymene	12.38±0.86
Eugenol	68.15±0.24
Geraniol	19.76±0.24
Geraniol Acetate	15.00±1.73
α-Humulene	10.18±0.63
Limonene	9.64±0.36
Linalool	14.29±0.10
Menthone	13.09±2.32
Myrcene	0.36±2.32
Nerol	0.33±0.17
β-Ocimene	14.73±2.23
α-Phellandrene	12.02±0.83
α-Pinene	13.45±1.04
β-Pinene	11.49±1.60
(+)-Sabinene	14.76±0.62
(-)-Thujone	6.70±1.52
(+)- Terpinen-4-ol	7.62±1.14
α-Terpinene	13.93±1.54
Terpinolene	6.61±1.25
γ-Terpinene	7.86±0.71
Thymol	68.75±0.27

Each value is the mean±SEM of three independent experiments.

The order of activity was determined as thymol > eugenol > carvacrol > citronellal > cedrene > geraniol > borneol > *cis*-3-hexan-1-ol > carvacrol methyl ether ~ geraniol acetate > (+)-sabinene > β-ocimene > linalool > α-terpinene > α-pinene > citral (*cis* + *trans*) > menthone > *p*-cymene > α-phellandrene > β-pinene > α-humulene > limonene > γ-terpinene > *trans*-β-caryophyllene > (+)-terpinen-4-ol > (-)-thujone > terpinolene > α-bisabolol > δ-3-carene > 1,8-cineole > aromadendrene > myrcene > nerol. The components carvacrol, eugenol and thymol demonstrated considerably more activity in this assay system than the remaining compounds, with equivalent antioxidant capacities of 58.93±2.79mmol L^{-1}, 68.15±0.24mmol L^{-1} and 68.75±0.27mmol L^{-1} respectively. Eugenol and thymol were found to be as effective as the synthetic antioxidants BHA and pyrogallol. Carvacrol demonstrated lower activity than BHA and pyrogallol but was found to be better than either BHT or α-tocopherol in this assay system. These antioxidants were marginally better than cedrene and citronellal and the moderately active components borneol, geraniol and *cis*-3-hexan-1-ol.

From data presented in Table 49, the following structure-activity characteristics/trends of the volatile oil constituents were evident from this study. The most active components were structures with a phenolic nucleus, e.g. carvacrol (**13**), eugenol (**26**) and thymol (**78**). While carvacrol methyl ether demonstrated some degree of activity, it was much lower than these components due to the loss of its free hydroxyl group. These components were significantly superior at trapping radicals generated in this assay system. Compounds with methyl-isopropyl cyclohexane rings with ketone functions were less active, e.g. menthone (**45**) and thujone (**76**), than non-carbonylated structures, e.g. sabinene (**62**). Monounsaturated cyclohexane rings were less active than polyunsaturated structures, e.g. the order of activity increased from terpinolene (**70**), terpinen-4-ol (**66**), limonene (**41**), α-phellandrene (**56**), *p*-cymene (**29**) to α-terpinene (**64**). Compounds with atypical terpene structures possessed little or low activity, e.g. aromadendrene (**3**) and 1,8-cineole (**22**). The α-isomer was more active than the β-isomer, e.g. α-pinene (**58**) and β-pinene (**59**), or the γ-isomer, e.g. α-terpinene (**64**) demonstrated pronounced activity relative to the low activity of γ-terpinene (**65**). The *cis*-isomer proved to be inactive contrary to the active *trans*-isomer, e.g. the active geraniol [(E)-3,7-dimethyl-2,6-Octadien-1-ol] (**33**) verses its *cis*-isomer nerol [(Z)-3,7-dimethyl-2,6-Octadien-1-ol] (**53**).

Nature of Volatile Oil Free Radical-Trapping Activity.

The activities of the volatile oils assayed in this study may be correlated to their respective constituents and their percentage composition, i.e. oils with high activity were composed of components which demonstrated pronounced activity when tested singularly or are generally considered to possess antioxidant properties (Aeschbach *et al.,* 1994; Deighton *et al.,* 1994a; Deighton *et al.,* 1994b; Schwarz *et al.*, 1996; Youdim, 1997).

The most potent oils clove, oregano and thyme possess high levels of phenolic substances. Clove volatile oil is principally composed of eugenol (87%) with significant minor quantities of β-caryophyllene and α-humulene, Table 11. Clearly from the data presented in Table 48, its potency is due to the high concentration of this phenylpropanoid and not β-caryophyllene and α-humulene, which were both poorly active. Similarly, in oregano and thyme volatile oils it is likely that their potency is significantly contributed to by the presence of both carvacrol and thymol and their high concentrations levels, 70% carvacrol and 48% thymol respectively. Furthermore, the component analysis of oregano (Table 20) and thyme (Table 21) reveals that the remaining principal components in these oils demonstrated moderate activity, such as *p*-cymene, limonene, linalool, α-pinene, α-terpinene and γ-terpinene.

The relatively modest activity of black pepper, which also contains eugenol (Table 10), may be explained in terms of the relative amounts of its components and their activity. Although eugenol was the strongest antioxidant component tested, it represents merely 5.56% of the oils composition, with the significantly less active constituent β-caryophyllene being found at much higher levels (24.21%). Consequently, the activity of black pepper appears to be due more to all the active components, e.g. δ-3-carene, β-caryophyllene, *p*-cymene, eugenol, α-humulene, limonene, α- and β-pinenes and α-phellandrene than one or two specific constituents.

The moderate activities of the volatile oils of geranium and the two nutmegs can also be explained by the activities of these oils main components. Geranium contains citronellal, β-caryophyllene, geraniol, linalool and menthone, Table 12. While the main components in the nutmegs are *p*-cymene, limonene, myrcene, myristicin, α- and β- pinenes, sabinene, α-terpinene, γ-terpinene, terpinen-4-ol and

α-thujene, Table 18 and Table 19. These compounds, present at relatively minor levels in these oils, were fairly moderate antioxidants (Table 49) which resulted in poor to moderate activity for these oils.

Melissa volatile oil demonstrated the least activity in this assay. However, in view of the component analysis for this oil (Table 16) and the activities of these components (Table 49), one would expect melissa to exert a greater capacity than the experimental data suggests (Table 48). Citronellal, geraniol, linalool, limonene, sabinene and α-pinene all demonstrated moderate to strong activity singularly with only the constituents myrcene (0.64%) and nerol (4.34%) expressing poor free radical-trapping activity. The poor antioxidant activity in this oil may be explained as for the other weak antioxidant oils in terms of poor activity of its constituent components or the low percentage composition of components with activity.

The data presented in Table 48 confirms that the volatile oils of clove and oregano possessed strong free radical-trapping activity on par with the synthetic antioxidants BHA and pyrogallol at 2.5mM. Thyme and black pepper possessed good to moderate free radical-trapping activity while geranium and nutmeg oils possessed less effective free radical-trapping activity. The data presented in Table 49 enabled an attempt to identify which components are responsible of the volatile oil activities shown in Table 48. The most active free radical-trapping compounds appeared to be carvacrol, eugenol and thymol which from GC and GC-MS component analyses (Chapter 2) were found to be present in the volatile oils of clove, oregano and thyme - the most active oils in this assay system. Similarly, oils with lower radical-trapping activities are composed principally of compounds that possessed lower radical-trapping activity or lack/contain minor amounts of the more active components such as carvacrol, eugenol or thymol.

5.4. Conclusions

The data presented in this study confirms that the volatile oils and specific components tested do possess varying degrees of antioxidant activities, comparable to synthetic or natural antioxidant substances currently utilized.

A clear potential use for these plant extracts or their constituents as antioxidants for use in food technology, delaying, retarding or preventing the oxidation process in the storage of raw materials or in food processing. Furthermore,

their antibacterial (Chapter 3) and antifungal (Chapter 4) properties enhances their appeal. To reduce lipid autoxidation it is necessary to control conditions and substances that promote oxidation. By selecting appropriate containers and packing materials and vacuum packaging, low oxygen uptake is maintained thereby eliminating oxygen during the manufacture and storage of foods. The use of good quality raw materials, packaging materials and appropriate processing techniques in the manufacturing process reduces endogenous oxidative activators, while food storage under cool and dark conditions retard rancidity by minimizing exogenous promoting factors. In practice these measures are rarely sufficient requiring the fortification of foodstuffs with antioxidants.

It is less clear from this study whether volatile oils or their components would be useful as antioxidant prophylactics. Although *in vitro* experiments generate data which may have predictive use *in vivo*, essential questions should be investigated before using these extracts for therapeutic use (Aruoma, 1994; Halliwell, 1990; Halliwell *et al.,* 1995). These include factors such as the bioavailability (absorption, distribution, metabolism and excretion), the availability at physiologically relevant concentration at the target site, the physiological condition of the recipient and the effect of nutrients and other drugs (Aruoma, 1996). Additionally, pro-oxidant activity should be assessed. Antioxidant substances capable of protecting lipids against peroxidation may have the potential to accelerate damage to intracellular components such as DNA, carbohydrates and proteins under certain conditions. Such studies, rarely carried out, would be an essential component in determining the toxicology of these secondary metabolites and confirming their *in vivo* utilization.

6. Effect of Dietary Supplementation of Plant Volatile Oils Upon Tissue Fatty Acid Composition in a Pregnant Rat Model

6.1. Introduction

The importance of long-chain polyunsaturated fatty acids and their metabolic precursors, the essential fatty acids, in mammalian tissues is associated with an extensive range of nutritional and metabolic functions including energy, growth, cellular metabolism and muscle activity (Dutta-Roy, 1994; Spector and Yorek, 1985; Sellmayer *et al.*, 1996). It has been hypothesized that the maternal, foetal and neonatal EFA/LCPUFA status is an important determinant of health and disease in infancy and later life (Dutta-Roy, 1997). Nutrition during pregnancy and early childhood has been demonstrated to affect the risk of neurodevelopmental disorders as well as psychomotor and cognitive development in infancy (Lucas *et al.*, 1992; Barker, 1993). Furthermore, evidence is rapidly growing that foetal and neonatal nutrition may have a programming effect upon the risk of cardiovascular and certain metabolic diseases in later life (Crawford, 1995; Fall *et al.*, 1995). In fact, EFAs and their LCPUFAs are of critical importance in foetal and neonatal development, due to their fundamental role as structural elements and functional modulators (Dutta-Roy, 1994; Spector and Yorek, 1985). It also has been shown, that ageing is associated with a marked decrease in PUFA levels in tissues with a resultant impact upon a number of key functions (Hall and Burdett, 1975; Wada and Tsumita, 1984; Pénzes *et al.*, 1988). The supply and incorporation of these cellular components in appropriate quantities for their physiological roles is determined by an array of counterbalancing factors, which is exacerbated under certain conditions. Perhaps the principal cause affecting a reduction in tissue levels of EFAs and LCPUFAs is lipid peroxidation.

The efficiency of the mammalian antioxidant defense system depends, in part, upon an adequate intake of foods such as fruits, vegetables and grain products containing antioxidants as such ascorbic acid, vitamin E, carotenoids and metal co-factors for specific antioxidant enzymes. Thus, the cellular antioxidant status can be

substantially influenced by the diet consumed. Therefore, dietary supplementation with known antioxidants should increase the protection of cellular sites susceptible to free radical-mediated oxidative damage, e.g. lipid-rich cellular membranes. Particular benefit would be evident under conditions of increased free radical-mediated tissue damage/oxidative stress and specific instances, e.g. pregnancy, where the overall maternal EFAs/LCPUFAs status steadily declines during the gestation period due to increasing placental and foetal requirements for these fatty acids; foetal development, where the supply and accumulation of sufficient quantities of EFAs/LCPUFAs during intrauterine life are of critical importance; neonatal growth, particularly in the case of premature infants which are known to have very marginal or deficient stores of many antioxidant compounds and experience relatively high free radical burden and therefore more oxidative stress than term infants or adults and in ageing.

Aromatic and medicinal plants have been implicated, although often empirically, with a range of beneficial qualities which have been exploited by man. These activities are often concentrated in the plant's volatile oil and have included antibacterial and antifungal properties. Recently, the screening of plant volatile oils has revealed that amongst these properties is often an ability to inhibit the peroxidative breakdown of lipid-rich systems, as the data presented in chapter 5 confirms. In view of these findings, the aims of this study were to investigate the effects of dietary incorporation of specific plant volatile oils on tissue fatty acids, with emphasis upon EFAs [linoleate and α-linolenate] and LCPUFAs [e.g. arachidonate, eicosapentænoate and docosahexænoate] levels, using a pregnant and neonatal animal model.

6.2. Materials and Methods

6.2.1. Materials

Where necessary, all reagents, chemicals and gases were of the highest purity obtainable as follows: Solvents: Rathburn Chemicals Ltd., Walkerburn, Scotland; Fisons Scientific Equipment, Loughborough, England; BDH, Lutterworth, England and Hayman Ltd., Witham, England. Reagents: Sigma Chemical, Poole, Dorset, England; Gases: BOC, Glasgow, UK. Basal diets were purchased from Special Diet Services (SDS), Essex, UK.

6.2.2. Feeding Trial Experimental Design

6.2.3. Protocol

A total of 32 pregnant, female rats were divided into 4 groups at random. All rats in each group were fed a standard diet, supplemented with oregano volatile oil, as described in Table 50, or clove and nutmeg volatile oils at 50μg g^{-1} *ad libitum* one week prior to and throughout pregnancy. In addition, rats were allowed unrestricted access to water. Diets were supplemented with the volatile oils by distribution onto the normal diet pellets dissolved in ethanol at a rate of 5mL Kg^{-1} diet. Diet A was treated with an equivalent amount of ethanol without any oregano volatile oil.

Neonatal rats were killed by an approved method within 48 hours of birth and samples were then frozen in liquid nitrogen immediately after removal and then stored until required for subsequent lipid analysis. At the same time, the mothers were anaesthetized using hypriam/midizolam (1.0mL 300g^{-1}b.wt.), a cardiac blood sample was taken and subsequently tissues were removed and frozen in liquid nitrogen immediately after removal and stored until required for subsequent lipid analysis.

Table 50. Diet Types Used in the Oregano Feeding Trial.

Diet	Dietary Description
Diet A	No Oregano volatile oil supplementation.
Diet B	Supplemented with oregano volatile oil at a level of 167 mg Kg^{-1}
Diet C	Supplemented with oregano volatile oil at a level of 334 mg Kg^{-1}
Diet D	Supplemented with oregano volatile oil at a level of 835 mg Kg^{-1}

6.2.4. Lipid Analysis

6.2.4.1. Extraction of Total Lipids

The total lipid associated with each organ was extracted with chloroform: methanol 2:1 (v/v) according to the method of Folch *et al* (1957), as follows. An

accurate amount of chopped tissue was weighed and placed into a small B24 test-tube. 20mL methanol was added followed by 20mL chloroform. The sample was then homogenized for 1 minute after which 20mL chloroform was added to give a final 2:1 methanol: chloroform ratio. The homogenated sample was left for half an hour before being filtered using a fluted Whatman Number 41 paper filter into a large B24 test-tube. Once the filtered solvent had passed through, any remaining solid material was rinsed with 2:1 chloroform: methanol and the filter paper was allowed to dry. To the filtrate, 12mL of 0.88% (w/v) potassium chloride was added and the solution was shaken vigorously, using a glass stopper to prevent any loss of sample. After releasing any pressure which may have built up, the aqueous and organic phases were allowed to separate for at least four hours.

After separation of the two phased had occurred, the upper aqueous layer was siphoned off and discarded. The lower organic phase was placed into a 100mL round bottomed flask to which two Pasteur pipette volumes of methanol was added then the organic solvent was removed by evaporation under vacuum using a rotary film evaporator at 50°C. After dissolution in chloroform, the residual was transferred qualitatively to a 10mL screw-cap vial which was then sealed under oxygen-free nitrogen and stored in the dark until analysis.

6.2.4.2. Determination of Lipid Content

The lipid extract (from the organs) in chloroform was transferred quantitatively to a 10mL volumetric flask and made up to the mark with chloroform. Using a bulb pipettes, 5mL of the solution was transferred to a pre-weighed 25mL round-bottomed-flask. The chloroform was removed using a rotary film evaporator and the flask containing the lipid was heated in an oven at 110°C over night to allow for complete removal of the solvent. After cooling, the flask was weighed and the total lipid weight determined from the difference between the empty and lipid-containing flask (gravimetric determination).

The remaining 5.0mL of sample was evaporated to dryness in a heating block at 50°C under a stream of oxygen-free nitrogen. 1mL chloroform (using a *500μL* syringe) was added to the extract and stored under oxygen-free nitrogen until required for lipid fraction separation and the determination of fatty acid composition.

6.2.4.3. Separation of Major Lipid Classes

Lipids were fractionated into their major classes by thin layer chromatography, on thin layer chromatoplates (20cm x 20cm) of 0.25mm silica gel G (Merck Ltd., Lutterworth, England) using a solvent system of hexane: diethyl ether: formic acid [80:20:1 (v/v/v)]. Due to the large number of TLC separations, plates where prepared as required.

6.2.4.3.1. Preparation of TLC Plates

20cm x 20cm glass supports were cleaned thoroughly with water and phosphate-free detergent, dried then finally cleansed with hexane. Five cleaned plates were placed in a commercial moving spreader (manufacturer unspecified) and secured flat into its frame. The absorbent was prepared by adding 50mL de-ionized water to 22.5g silica gel G which was then shaken vigorously to ensure a homogenous slurry. This mixture was then poured into a hopper, with its trailing face set at 0.25mm. The hopper was then pushed across the plates with an steady and even motion. The frame was then gently 'banged' to ensure an even film of absorbent across the plates and left to dry at room temperature.

After the freshly prepared plates had dried, they were placed in a drying oven at 110°C over night to activate or until required. Plates were removed from the oven 30 minutes before use in order to enable them to cool down.

6.2.4.3.2. Spotting and Running of TLC Plates

Before the lipid sample was applied to the TLC plates, the sides were cleaned using paper towel so as to prevent the applied lipid sample from being flushed off the TLC plate while developing. The plate was then divided into equal half so that two separate samples could be run on each plate.

Using a glass syringe, between 50μL - 250μL sample was applied across the bottom of each TLC plate in a line no thicker than 3mm, approximately 2.0cm from the bottom edge and 0.5cm from the sides. Before applying any sample over a previously loaded area, care was taken to ensure that the silica gel had absorbed the sample and had dried. After spotting the plates, they were then placed vertically into the chromatank as quickly as possible to enable a uniform solvent front. An absorbent towel was placed in the chromatank before the solvent system was added

to aid the saturation of the tanks atmosphere by acting as a wick. The tank was then sealed and allowed to equilibrate for 30 minutes

Once the solvent front had reached approximately 1.0cm from the top of the TLC plate, the chromatank was opened and the plates were removed and allowed to dry before the processes required of visualization of the separated lipid fractions.

6.2.4.4. Visualising TLC plates and Elution of Different Lipid Fractions from the TLC Plates

After the developed plates had dried, the separated lipid fractions were lightly sprayed with a 0.1% (w/v) solution of 2,7-dichlorofluorescein in methanol using a sprayer bottle powered by an aerosol cartridge. Bands corresponding to cholesteryl ester, triacylglyceride, free fatty acid and phospholipid fractions were visualized using an ultraviolet.

Each band was removed from the plate by scraping it onto glossy paper with a flat-ended spatula then placed into a centrifuge tube. The cholesteryl ester, triacylglyceride, free fatty acid and cholesterol lipid fractions were re-dissolved using two Pasteur pipette volumes of diethyl ether, while the phospholipid rich band was re-dissolved using two Pasteur pipette volumes of methanol. In each case, the tubes were centrifuged to sediment the silica gel and the lipid-containing solvent was transferred to a 50mL round-bottomed flask by decantation for fatty acid composition determination.

6.2.4.5. Determination of Fatty Acid Composition

6.2.4.5.1. Transmethylation of Fatty Acids

Fatty acid methyl esters were generated by refluxing the lipid fractions with dry methanolic sulphuric acid (methanol: toluene: concentrated sulphuric acid [20:10:1 (v/v/v)]). In all cases a pentadecanoic acid (15:0) internal standard in methanol was added to 50mL flasks containing the lipid fractions, 0.322mg L^{-1} 15:0 being added to triacylglyceride and phospholipid and 0.0322mg Kg^{-1} 15:0 being added to cholesteryl ester and free fatty acid fractions before being evaporated to dryness, using a rotary evaporator. Once the lipids were freed of solvent, 4mL methylating agent was added and the flasks were swirled before being placed on a

heating range at 50°C and refluxed for 45 minutes. After refluxing, the flasks were allowed to cool for 15 minutes before 10mL de-ionized water and 10mL hexane were added. The flasks were shaken vigorously to allow full extraction of fatty acid methyl esters into the hexane phase (top layer) then the contents were transferred into B19 test tubes. The hexane layer was transferred using a glass Pasteur pipette into further B19 test-tubes and the residual water removed by the addition of approximately 10g anhydrous sodium sulphate: sodium hydrogen carbonate [4:1 (w/w)]. After a period no less then 30 minutes, the methyl ester containing hexane was transferred into B14 test tubes until ready for concentration for GC injection.

6.2.4.6. Determination of Fatty Acids by Gas Chromatography

6.2.4.6.1. Concentration of Fatty Acid Samples for Injection

The hexane containing methyl esters B14 test-tubes were placed in a heating block at 50°C and evaporated to dryness under a stream of oxygen-free nitrogen gas. Once dry, the methyl esters were taken up in volumes of hexane appropriate to their amount then transferred to GC vials and capped to minimize loss through evaporation before GC analysis.

6.2.4.6.2. Gas Chromatography Analysis

Fatty acid methyl esters were separated using a Model 428 Gas Chromatograph Instrument (Canberra-Packard Instruments Ltd., Berkshire, England) using a 30m x 0.25μm film Carbowax capillary column (Econo-Cap, Alltech UK Ltd., Camforth, UK.) by injecting the sample via a CP9010 Autosampler (Chrompack, London, England). The relative proportions of the fatty acids (% w/w of total fatty acids) were quantified by integration of the amplified signal using an EZ-Chrom™ Data Handling System (Soeck Analytical, Alloa, UK).

6.3. Results

6.3.1. The Effect of the Oral Administration of Oregano Volatile Oil on the Lipid Composition of Pregnant Rats and Their Neonates.

6.3.1.1. Maternal Adipose Tissue

The effect of the supplementation of oregano volatile oil, as described in the Materials and Methods (Section 6.2.), upon the fatty acid composition of the TAG fractions from maternal adipose tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6:n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 17.

The principal FAs in the TAG fraction were the saturates 14:0, 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9*, and 18:1*n-7*; the dienoic 18:2*n-6* *n-6* PUFA and the trienoic 18:3*n-3* and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 17. The proportion of total saturates decreased 2.45%, 10.1% and 6.14% whereas the proportion of total monounsaturates increased 12.9% at 167mg Kg^{-1} and 6.1% at 334mg Kg^{-1} and decreased 5.1% decrease at the 834mg Kg^{-1} treatment level. The proportion of total *n-6* PUFAs decreased 7.6% and 4.1% at the first two concentration levels and increased 4.7% at the highest level. In the case of the proportion of total *n-3* PUFAs, the oregano treatment levels caused decreases of 26.8%, 20.0% and 12.0% in this fraction. The effect of these changes upon the - proportion of total PUFAs resulted in a 10.1% and 6.2% decrease at 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels and a 2.6% increase at 834mg Kg^{-1}. The unsaturate: saturate ratios increased 16.0%, 18.0% and 1.0% respectively across the treatment levels while the polyunsaturate: saturate ratio decreased 7.6% at 167mg Kg^{-1} and increased 4.3% and 9.2% over the remaining concentrations. The *n-6:n-3* ratio remained at increased levels across the treatment concentrations with increases of 25.4% at 167mg Kg^{-1}, 20.1% at 334mg Kg^{-1} and 19.1% at 834mg Kg^{-1}.

There were no significant changes in individual saturates while the monoenoic acid 18:1*n-6* increased 12.9% ($P<0.01$) at 167mg Kg^{-1} and 7.8% ($P<0.01$) at 334mg Kg^{-1}, 18:1*n-7* increased 21.5% ($P<0.01$) at 167mg Kg^{-1} and 20:1*n-9*

decreased 47.2% ($P<0.05$) at the 167mg Kg^{-1} treatment level. In the case of *n-6* PUFAs, these acids tended to decrease, principally at 167mg Kg^{-1} whilst the decreases at 334mg Kg^{-1} were principally restricted to tetrænoic acids: 18:3*n-6*, 20:2*n-6*, 20:3*n-6*, 20:4*n-6* and 22:4*n-6* decreased 30.8% ($P<0.01$), 34.5% ($P<0.01$), 42.9% ($P<0.05$), 35.6% ($P<0.01$) and 26.3% ($P<0.01$) respectively at 167mg Kg^{-1} while 20:4*n-6* and 22:4*n-6* decreased 20.5% ($P<0.05$) and 21.1% ($P<0.01$) respectively at 334mg Kg^{-1}. Similarly, significant changes in *n-3* PUFAs included 22:5*n-3* which decreased 26.5% ($P<0.05$), 23.5% ($P<0.05$) and 19.2% ($P<0.05$) respectively and 22:6*n-3* which decreased 32.1% ($P<0.01$) at 167mg Kg^{-1}, 27.5% ($P<0.001$) at 334mg Kg^{-1} and 18.9% ($P<0.05$) at 834mg Kg^{-1} concentration levels.

6.3.1.2. Maternal Brain Tissue

The effect of the supplementation of oregano volatile oil, as described in the Materials and Methods (Section 6.2.), upon the fatty acid composition of the PL fractions from maternal brain tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6:n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 18.

The principal FAs in the PL fraction were the saturates 16:0 and 18:0; the monounsaturates 17:1*n-7*, 18:1*n-9*, 18:1*n-7* and 20:1*n-9*, the tetrænoic 20:4*n-6* and 22:4*n-6* *n-6* PUFAs and the hexænoic 22:6*n-3* *n-3* PUFA, Appendix 18. The proportion of total saturates remained at their baseline level at 167mg Kg^{-1} and increased from 2.5% at 334mg Kg^{-1} to 6.6% at 834mg Kg^{-1}. In the case of the proportion of total monounsaturates, the oregano treatments caused a reduction in the these values relative to their baseline level, with decreases of between 1.9% at 167mg Kg^{-1} to 7.5% at 834mg Kg^{-1} respectively. In the case of the proportion of total *n-6* and *n-3* PUFAs, the oregano treatment levels caused similar effect upon these proportions with only minor fluctuations at the tested concentration levels. Consequently, the effect of the treatments upon the proportions of the total PUFAs were very minor, with an increase of 2.9% at 167mg Kg^{-1} and decreases of 1.8% at the remaining concentration levels. The unsaturate: saturate ratios were lower than the baseline with decreases of 1.3%, 7.6% and 12.7% at 167mg Kg^{-1}, 334mg Kg^{-1}

and 834mg Kg^{-1} and although the polyunsaturate: saturate ratio at 167mg Kg^{-1} increased 2.9% the remaining ratios decreased between 4.4% and 8.8% at 334mg Kg^{-1} and 834mg Kg^{-1} respectively. The effect upon the *n-6:n-3* PUFA ratios of the changes in the *n-6* and *n*-3 PUFAs resulted in increases of 1.1% at both 167mg Kg^{-1} and 334mg Kg^{-1} while at 834mg Kg^{-1}, there was a 9.0% increase in this ratio.

There were no significant changes in individual saturates in this fraction whilst the monoenoic acid 17:1*n-7* decreasing 74.1% ($P<0.001$) at 334mg Kg^{-1} and 20:1*n-9* decreased 10.3% ($P<0.01$) and 37.3% ($P<0.001$) at 334mg Kg^{-1} and 834mg Kg^{-1} treatment levels. There were no significant changes in individual *n-6* PUFAs except 20:2*n-6*, which decreased to below trace, levels ($P<0.001$) at 834mg Kg^{-1}. However, in the case of individual *n-3* PUFAs, significant changes were restricted to 18:3*n-3* which increased 50.0% ($P<0.01$) and 35.7% ($P<0.05$) at 334mg Kg^{-1} and 834mg Kg^{-1} and 22:5*n-3* which dramatically increased 230.0% ($P<0.001$) at 834mg Kg^{-1}.

6.3.1.3. Maternal Heart Tissue

The effect of the supplementation of oregano volatile oil, as described in the Materials and Methods (Section 6.2.), upon the fatty acid composition of the FFA and PL fractions from maternal heart tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6:n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 19 and Appendix 20.

The principal FAs in the FFA fraction were the saturates 16:0 and 18:0; the monounsaturates 18:1*n-9* and 18:1*n-3*, the dienoic 18:2*n-6* and 20:2*n-6* and tetrænoic 20:4*n-6* *n-6* PUFAs and the pentænoic 22:5*n-3* and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 19. The proportions of total saturates increased 5.1% and 6.5% at 167mg Kg^{-1} and 834mg Kg^{-1} but decreased 1.7% at 334mg Kg^{-1} while the proportion of total monounsaturates were consistently higher than the baseline level, with increases ranging from 16.1%, 22.1% and 25.6% across the concentration levels. Both the proportion of total *n-6* PUFAs and *n-3* PUFAs decreased 5.9%, 10.7% and 10.4% and 14.9%, 11.5% and 6.0% respectively at 167mg Kg^{-1}, 334mg Kg^{-1} and 834mg Kg^{-1}. The result of the changes in the proportion of *n-6* and *n-3* PUFAs

caused minor decreases of 0.7%, 5.0% and 6.4% in the proportions of total PUFAs across the concentration levels. The unsaturate: saturate ratios increased 15.6%, 28.1% and 9.4% with increasing treatment levels while the polyunsaturate: saturate ratios tended to be lower that the baseline level, decreasing 5.4%, 2.7% and 12.6%. The result of the changes in the *n-6* and *n-3* PUFAs caused the ratios of *n-6:n-3* PUFAs to decrease 18.2%, 19.9% and 15.4% across the concentration levels.

There were no significant changes in individual saturates, except at 834mg Kg^{-1} where 20:0 decreased to below trace levels ($P<0.001$), or individual monounsaturates. Significant changes in individual *n-6* PUFAs were limited to the 18:3*n-6* which increased 57.1% ($P<0.01$) at 167mg Kg^{-1} and 42.9% ($p<0.05$) across the remaining concentration levels and 20:2*n-6* which increased 62.0% ($P<0.05$) and 35.4% ($P<0.05$) with 167mg Kg^{-1} and 834mg Kg^{-1} treatments. The oregano treatments did not cause any significant changes in individual *n-3* PUFAs in this fraction.

The principal FAs in the PL fraction extracted from the heart tissues were the saturates 16:0 and 18:0; the monoenoic acids 18:1*n-9* and 18:1*n-3*; the dienoic 18:2*n-6* and tetrænoic 20:4*n-6* *n-6* PUFAs and the pentænoic 22:5*n-3* and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 20. The proportions of total saturates decreased 6.9%, 4.2% and 1.7% and monounsaturates decreased 11.7%, 9.8% and 6.1% across the concentration levels. This trend was not repeated in the case of the proportion of *n-6* or *n-3* PUFAs. At 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels, the proportions of total *n-6* PUFAs increased 1.5% and 0.2% respectively whilst at 834mg Kg^{-1} there was a marginal 6.1% decrease relative to the baseline level.

In the case of the proportions of total *n-3* PUFAs, large increases occurred ranging 17.4% at 167mg Kg^{-1} to 22.3% at 834mg Kg^{-1}. The result of these changes upon the proportion of total PUFAs caused increases of 7.6%, 5.9% and 2.6% relative to the baseline level. The treatment levels produced little change in the unsaturate: saturate ratios, with the treatments causing minor decreases of 4.8% at 167mg Kg^{-1} and 334mg Kg^{-1} but having no effect at 834mg Kg^{-1}. The polyunsaturate: saturate ratios were affect to a greater extent at these treatment levels, with increases of 14.9% at 167mg Kg^{-1}, 10.2% at 334mg Kg^{-1} and 3.9% at 834mg Kg^{-1}. However, the *n-6:n-3* ratios were lower than the baseline level with decreases of 15.4% at 167mg Kg^{-1}, 14.9% at 334mg Kg^{-1} and 8.2% at 834mg Kg^{-1}.

Significant changes in individual saturates were limited to 18:0 which increased 10.3% ($P<0.05$) at 334mg Kg^{-1} and 20:0 which increased 20.0% ($P<0.05$) at 834mg Kg^{-1} while in the case of monoenoic acids, 17:1*n-7* and 20:1*n-9* increased 51.5% ($P<0.05$) and 35.7% ($P<0.05$) at 834mg Kg^{-1} and 167mg Kg^{-1} respectively. Significant changes is *n-6* PUFAs were restricted to 18:2*n-6* which decreased 17.6% ($P<0.05$) at 834mg Kg^{-1} while the *n-3* PUFA 22:5*n-3* increased of 23.5% ($P<0.05$) and 22.6% ($P<0.05$) at 168mg Kg^{-1} and 334mg Kg^{-1} respectively.

6.3.1.4. Maternal Kidney Tissue

The effect of the supplementation of oregano volatile oil, as described in the Materials and Methods (Section 6.2.), upon the fatty acid composition of the TAG, FFA and PL fractions from maternal kidney tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6:n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 21 to Appendix 23.

The principal FAs in the TAG fraction were the saturates 14:0, 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-7*, the dienoic 18:2*n-6* and tetrænoic 20:4*n-6* *n-6* PUFAs and the trienoic 18:3*n-3* *n-3* PUFA, in Appendix 21. The proportion of total saturates and monounsaturates varied little from their respective baseline levels, with the proportion of total saturates increasing 1.2% at 334mg Kg^{-1} and decreasing 3.4% at 834mg Kg^{-1} whilst the proportion of total monounsaturates increased 7.1% at 334mg Kg^{-1} and decreased 1.9% at 834mg Kg^{-1}. Similar trends occurred in the case of the proportion of total *n-6* and *n-3* PUFAs, with total *n-6* PUFAs decreasing 5.9% at 167mg Kg^{-1}, 10.1% at 334mg Kg^{-1} and increasing 0.7% at 834mg Kg^{-1} whilst the total *n-3* PUFAs decreased 10.9% at 334mg Kg^{-1} and increased 15.7% at 834mg Kg^{-1}. The effect of these changes upon the proportion of total PUFAs were to cause decreases of 5.4% and 10.5% at 167mg Kg^{-1} and 334mg Kg^{-1} respectively and a 1.6% increase at 834mg Kg^{-1}. The oregano treatment levels had little effect upon the unsaturate: saturate ratios, with minor increases occurring at 334mg Kg^{-1} and 834mg Kg^{-1} - 5.4% and 0.9% respectively. Similar, in the case of the polyunsaturate: saturate and *n-6:n-3*PUFA ratios there were minor changes, with the polyunsaturate: saturate ratio decreasing 5.2% and 11.5% at 167mg Kg^{-1} and 334mg

Kg^{-1} whilst increasing 5.2% at the highest concentration level while the *n-6:n-3* PUFA ratio decreasing 7.5% and 13.1% at 167mg Kg^{-1} and 834mg Kg^{-1} and increasing 1.0% at 334mg Kg^{-1}.

There were no significant changes in individual saturates in this fraction except 18:0 and 20:0 which increased 19.0% ($P<0.01$) and 33.3% ($P<0.05$) at 834mg Kg^{-1} respectively. Significant changes in the monoenoic acids were restricted to 16:1*n-7* and 20:1*n-9* which increased 19.7% ($P<0.01$) and 93.3% ($P<0.01$) at 334mg Kg^{-1} and 167mg Kg^{-1}. Significant changes in individual *n-6* PUFAs were restricted to 18:2*n-6* which decreased 9.5% ($P<0.05$) at 334mg Kg^{-1}, 18:3*n-6* which increased 63.6% ($P<0.01$), 20:2*n-6* which increased from below trace levels to 0.39±0.06 ($P<0.001$) and 0.40±0.08 ($P<0.001$) at 167mg Kg^{-1} and 834mg Kg^{-1} respectively, 20:3*n-6* which increased 22.02% ($P<0.05$), 20:4*n-6* which increased 47.7% ($P<0.01$) and 22:4*n-6* which decreased 23.1% ($P<0.05$). Significant changes in individual *n-3* PUFAs included the PUFAs 18:3*n-3* which decreased 22.5% ($P<0.01$) and 20:5*n-3* which increased 78.6% ($P<0.01$) and 128.6% ($P<0.001$) at 167mg Kg^{-1} and 834mg Kg^{-1} respectively.

The principal FAs in the FFA fraction were the saturates 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and tetrænoic 20:4*n-6* *n-6* PUFAs and the pentænoic 20:5*n-3* and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 22. The proportion of total saturates increased 5.7%, 4.4% and 3.8% whilst the proportion of total monounsaturates decreased 12.0%, 6.2% and 13.9% at 167mg Kg^{-1}, 334mg Kg^{-1} and 834mg Kg^{-1} respectively. The proportion of total *n-6* PUFAs decreased 3.3%, 1.8% and 0.8% while the proportion of total *n-3* PUFAs increased 4.1%, 6.0% and 11.0% at 167mg Kg^{-1}, 334mg Kg^{-1} and 834mg Kg^{-1} respectively. These changes resulted in the proportion of total PUFAs to decrease 1.8% and 0.4% at 167mg Kg^{-1} and 334mg Kg^{-1} but increased 1.4% at 834mg Kg^{-1}. The effect of the administration of oregano at these treatment levels upon the unsaturate: saturate ratios, polyunsaturate: saturate and *n-6: n-3* PUFA ratios were to reduce these indices to below their respective baseline levels.

There were no significant changes in individual saturates in this fraction whilst significant changes were restricted to the monoenoic acids 17:1*n-7* which decreased 120.0% ($P<0.001$), 53.3% ($P<0.01$) and 46.6% ($P<0.01$) at 167mg Kg^{-1}, 334mg Kg^{-1} and 834mg Kg^{-1} respectively and both 18:1*n-7* and 20:1*n-9* which

decreased 14.6% ($P<0.05$) and 24.5% ($P<0.05$) respectively at 834mg Kg^{-1}. There were no significant changes in the *n-6* PUFAs or *n-3* PUFAs except 18:3*n-3* which 40.5% ($P<0.05$) and 30.4% ($P<0.05$) at 167mg Kg^{-1} and 334mg Kg^{-1} and 22:5*n-3* which increased 38.9% ($P<0.01$) at 167mg Kg^{-1} and 27.4% ($P<0.01$) 334mg Kg^{-1}.

The principal FAs in the PL fraction extracted from the kidney tissues were the saturates 16:0 and 18:0; the monounsaturates 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and tetrænoic 20:4*n-6* *n-6* PUFAs and the hexænoic 22:6*n-3* *n-3* PUFA, Appendix 23. The proportion of total saturates decreased 3.6%, 3.0% and 3.4% while the total monounsaturates decreased 1.4%, 4.1% and 9.6% at 167mg Kg^{-1}, 334mg Kg^{-1} and 834mg Kg^{-1} respectively. The treatments resulted in the proportion of total *n-6* PUFAs increasing 5.8%, 0.9% and 4.9% at 167mg Kg^{-1}, 334mg Kg^{-1} and 834mg Kg^{-1} while the proportion of total *n-3* PUFAs increased 23.9%, 20.7% and 15.6% respectively across the concentration levels. These changes resulted in increases of 8.4%, 3.7% and 6.5% in the proportion of total PUFAs at 167mg Kg^{-1}, 334mg Kg^{-1} and 834mg Kg^{-1}. The unsaturate: saturate ratios did not fluctuate from the baseline level with the treatment concentration levels, however there were large variations in the polyunsaturate: saturate ratios with increases of 12.5%, 7.5% and 11.3% across the oregano levels. The *n-6:n-3* PUFAs ratios were lower than the baseline levels in this fraction, with decreases of 14.6%, 17.0% and 9.2% across the concentration levels.

There were no significant changes in individual saturates except a 11.6% ($P<0.05$) decrease in 17:0 at 334mg Kg^{-1}. Similarly, in the case of individual monoenoic acids, *n-6* PUFAs or *n-3* PUFAs in this fraction.

6.3.1.5. Maternal Liver Tissue

The effect of the supplementation of oregano volatile oil, as described in the Materials and Methods (Section 6.2.), upon the fatty acid composition of the CE, TAG, FFA and PL fractions from maternal liver tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6:n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 24 to Appendix 27.

The principal FAs in the CE fraction were the saturates 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-7*, the dienoic 18:2*n-6* and 20:2*n-6* and tetrænoic 20:4*n-6* *n-6* PUFAs and the trienoic 18:3*n-3*, pentænoic 20:5*n-3* and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 24. The proportion of total saturates increased 11.2%, 16.5% and 10.9% while the proportion of total monounsaturates increased 32.5% and 12.8% at 167mg Kg^{-1} and 834mg Kg^{-1}. The proportion of the total *n-6* PUFAs decreased 6.9% and 7.1% at 167mg Kg^{-1} and 334mg Kg^{-1} while the proportion of total *n-3* PUFAs decreased 29.4%, 18.1% and 10.2%. The effect of these changes upon the proportion of total PUFAs caused decreases of 12.8%, 10.2% and 7.2% across the concentration levels.

The effect of the oregano treatment levels upon the unsaturate: saturate ratios were to cause a 20.0% and 2.9% increases at 164mg Kg^{-1} and 834mg Kg^{-1} while causing a 14.3% decrease at 334mg Kg^{-1}. In the case of the polyunsaturate: saturate ratios, the treatment levels resulted in decreases of 21.9%, 22.6% and 16.4% across the concentrations levels. However, the *n-6:n-3* ratios increased by 32.1%, 13.6% and 43.0%.

There were no significant changes in individual saturates in this fraction. However there were significant changes in the monoenoic acids 16:1*n-7* which increased 34.6% ($P<0.01$) at 167mg Kg^{-1}, 57.9% ($P<0.01$) at 334mg Kg^{-1} and 52.4% ($P<0.01$) at 834mg Kg^{-1} and 17:1*n-7* decreased 48.0% ($P<0.01$) and 20.0% ($P<0.05$) at 334mg Kg^{-1} and 834mg Kg^{-1}. As in the case of the saturates, there were no significant changes in individual *n-6* PUFAs with the treatment levels. In the case of individual *n-3* PUFAs 22:5*n-3* decreased 31.1% ($P<0.05$) at 167mg Kg^{-1} and 334mg Kg^{-1} and 38.1% ($P<0.05$) at 834mg Kg^{-1} while 22:6*n-3* decreased 41.1% ($P<0.01$), 35.6% ($P<0.05$) and 34.4% ($P<0.05$) at 167mg Kg^{-1}, 334mg Kg^{-1} and 834mg Kg^{-1} respectively.

The principal FAs in the TAG fraction were the saturates 16:0 and 18:0, the monoenoic acids 16:1*n-7*, 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and 20:4*n-6* and tetrænoic 22:4*n-6* *n-6* PUFAs and the trienoic 18:3*n-3*, pentænoic 22:5*n-3* and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 25. The proportion of total saturates decreased 7.1% and 7.4% at 167mg Kg^{-1} and 334mg Kg^{-1} and increased 4.3% at 834mg Kg^{-1} whilst the proportion of total monounsaturates increased 12.8% and 12.3% at 167mg Kg^{-1} and 334mg Kg^{-1} but decreased 12.3% at 834mg Kg^{-1}. In the

case of the proportion of total *n-6* PUFAs and *n-3* PUFAs, the *n-6* PUFAs decreased 3.0% at 167mg Kg^{-1} and increased 6.6% and 4.4% at 334mg Kg^{-1} and 834mg Kg^{-1} while the *n-3* PUFAs decreased 43.7%, 29.2% and 20.1% at 167mg Kg^{-1}, 334mg Kg^{-1} and 834mg Kg^{-1} respectively. As a result of these changes, the proportion of total PUFAs decreased 18.5%, 6.9% and 4.7% across the concentration levels. The effect of the treatment levels upon the unsaturate: saturate ratios were to increases of 21.7% at 167mg Kg^{-1} and 334mg Kg^{-1} but decreased 4.9% at 834mg Kg^{-1} while the polyunsaturate: saturate ratios consistently were lower than the baseline level across the concentration levels. The effect upon the *n-6:n-3* ratios of the treatments were to cause increases of 72.2%, 50.3% and 30.8% across the treatment levels.

There were no significant changes in individual saturates except for 18:0 which decreased 25.1% ($P<0.05$) and 46.1% ($P<0.01$) at 167mg Kg^{-1} and 334mg Kg^{-1} while the monounsaturate 16:1*n-7* increased 97.9% ($P<0.001$) at 167mg Kg^{-1}, 52.1% ($P<0.01$) at 334mg Kg^{-1} and 117.7% ($P<0.001$) at 834mg Kg^{-1}. In the case of individual *n-6* PUFAs, significant changes occurred in 18:3*n-6* which increased increasing 100% ($P<0.001$) at 167mg Kg^{-1} and 50.0% ($P<0.01$) at 334mg Kg^{-1}; 20:2*n-6* which increased 233.0% ($P<0.001$) and 150.0% ($P<0.001$) at 167mg Kg^{-1} and 834mg Kg^{-1} respectively and 22.4*n-6* which decreasing 65.4%, 53.5% and 75.6% at $P<0.01$ significance level, across the treatments. The *n-3* PUFA 20:5*n-3* increased 90.8% ($P<0.001$) and 92.1% ($P<0.001$) at 334mg Kg^{-1} and 834mg Kg^{-1} while 22:6*n-3* decreased 53.2% ($P<0.01$) and 45.7% ($P<0.01$) at 167mg Kg^{-1} and 334mg Kg^{-1} respectively while at 834mg Kg^{-1} decreased 25.3% ($P<0.05$).

The principal FAs in the FFA fraction were the saturates 16:0 and 18:0, the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-7* the dienoic 18:2*n-6* and tetrænoic 20:4*n-6* *n-6* PUFAs and the trienoic 18:3*n-3*, pentænoic 22:5*n-3* and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 26. The proportion of total saturates decreased 22.2%, 50.4% and 15.2% while the proportion of total monounsaturates increased 62.1%, 37.1% and 33.4%. The proportion of total *n-6* PUFAs increased 4.0%, 20.1% and 19.7% whilst the proportion of total *n-3* PUFAs decreased 23.7% at 167mg Kg^{-1} and 22.7% across the remaining concentration levels. The effect of these changes upon the proportion of total PUFAs caused a 7.9% decrease at 167mg Kg^{-1} and a minor increase of 1.7% at the remaining levels. The unsaturate: saturate, polyunsaturate: saturate and *n-6: n-3* ratios all increased with the oregano treatment levels. The

unsaturate: saturate ratios increased 107.4%, 177.8% and 59.3% while the polyunsaturate: saturate ratios increased 18.2%, 104.5% and 19.1%. The *n-6:n-3* ratios increased 36.4%, 56.1% and 55.3% respectively.

Significant changes in individual saturates were restricted to 18:0 which decreased 43.3%, 50.7% and 33.5% at $P<0.01$ level of significance whilst the monoenoic acid 17:1*n-7* which increased 85.7% ($P<0.001$) at 167mg Kg^{-1} and 128.6% ($P<0.001$) at 334mg Kg^{-1} and 18:1*n-9* which increased 75.3% at 167mg Kg^{-1}. Significant changes in *n-6* PUFAs were restricted to 18:3*n-6* which increased 83.3% ($P<0.01$) and 75.0% ($P<0.01$) at 167mg Kg^{-1} and 334mg Kg^{-1} respectively and 20:2*n-6* which increase from a baseline value 0.08±0.02 to 0.40±0.06 ($P<0.001$) at 167mg Kg^{-1} and 0.43±0.04 ($P<0.001$) at 834mg Kg^{-1}. The *n-3* PUFA 18:3*n-3* increased 71.3% at 334mg Kg^{-1} ($P<0.01$) whilst 20:5*n-3* increased 97.4% ($P<0.001$) at 334mg Kg^{-1}. 22:6*n-3* decreased 31.1% ($P<0.05$), 38.5% ($P<0.01$) and 29.5% ($P<0.05$) across the treatment levels.

The principal FAs in the PL fraction were the saturates 16:0 and 18:0; the monounsaturates 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and tetrænoic 20:4*n-6 n-6* PUFAs and the pentænoic 22:5*n-3* and hexænoic 22:6*n-3 n-3* PUFAs, Appendix 27. The proportion of total saturates increased 2.6% and 1.8% at 167mg Kg^{-1} and 834mg Kg^{-1} but decreased 2.0% at 334mg Kg^{-1}. Whilst the proportion of total monounsaturates increased 35.4%, 14.6% and 16.6% across the concentration levels. A similar trend occurred in the proportion of total *n-6* PUFAs, with increases of 26.3% and 10.6% at 167mg Kg^{-1} and 834mg Kg^{-1} respectively. The proportion of total *n-3* PUFAs decreased 24.1% and 12.9% at 167mg Kg^{-1} and 834mg Kg^{-1} respectively. The effect of these changes upon the proportion of total PUFAs at each treatment was minimal with 1.6% and 2.4% decreases 167mg Kg^{-1} and 834mg Kg^{-1} and a minor increase at 334mg Kg^{-1} of 0.4%. The oregano treatment levels caused the unsaturate: saturate ratios to increase 33.3%, 22.2% and 11.1% whilst producing 3.6% decreases at 167mg Kg^{-1} and 834mg Kg^{-1} concentration levels in the polyunsaturate: saturate ratios. The *n-6: n-3* ratios increased 66.7% at 167mg Kg^{-1} and 27.2% at 834mg Kg^{-1} whilst at 334mg Kg^{-1} the ratio remained at baseline levels.

Significant changes in individual saturates occurred in the case of 14:0 which increased 50.0% ($P<0.001$) at 167mg Kg^{-1} and 834mg Kg^{-1} concentration levels and 33.3% ($P<0.01$) at 334mg Kg^{-1} while 17:0 increased 42.2% ($P<0.05$) at 167mg Kg^{-1}.

Significant changes in individual monoenoic acids included 16:1*n-7* which increased 46.2% ($P<0.05$), 24.6% ($P<0.05$) and 53.8% ($P<0.05$) and 18:1*n-9* which increased 56.3% ($P<0.001$), 31.6% ($P<0.05$) and 25.7% ($P<0.05$) across the concentration levels. Significant changes in individual *n-6* PUFAs included 18:*2n-6* which increased 35.4% ($P<0.01$) at 167mg Kg^{-1}, 18:3*n-6* which increased 62.5% ($P<0.001$) and 25.0% ($P<0.05$) at 167mg Kg^{-1} and 334mg Kg^{-1} and 20:3*n-6* which increased from 0.26±0.02 to 0.82±0.08 ($P<0.001$) at 167mg Kg^{-1}. Similarly, the *n-3* PUFAs which significantly changed with the oregano treatment levels included 18:3*n-6* which increased 37.5% ($P<0.01$), 50.0% ($P<0.05$) and 37.5% ($P<0.01$) across the concentration levels, 20:5*n-3* which increased from 0.22±0.01 to 0.65±0.09 ($P<0.001$) at 167mg Kg^{-1}, 22:5*n-3* which decreased 24.8% ($P<0.05$) at 834mg Kg^{-1} and 22:6*n-3* which decreased 28.5% ($P<0.01$) at 167mg Kg^{-1}.

6.3.1.6. Neonatal Brain Tissue

The effect of the supplementation of oregano volatile oil, as described in the Materials and Methods (Section 6.2.), upon the fatty acid composition of the PL fractions from neonatal brain tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6:n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 28.

The principal FAs in the PL fraction were the saturates 14:0, 16:0 and 18:0; the monounsaturates 16:1*n-7*, 17:1*n-7*, 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and tetrænoic 20:4*n-6* and 22:4*n-6* *n-6* PUFAs and the hexænoic 22:6*n-3* *n-3* PUFA, Appendix 28. The proportions of total saturates decreased 1.1% and 3.7% at 167mg Kg^{-1} and 334mg Kg^{-1} while increased 2.6% at 834mg Kg^{-1}. Similarly, the proportion of total monounsaturates decreased 8.1% and 6.0% at 167mg Kg^{-1} and 334mg Kg^{-1} while increased 0.5% at 834mg Kg^{-1}. The proportion of total *n-6* PUFAs increased marginally by 9.1% at 167mg Kg^{-1} and 0.7% at 334mg Kg^{-1} whilst the proportion of total *n-3* PUFAs decreased 0.8% and 1.6% at 167mg Kg^{-1} and 834mg Kg^{-1} but increased 3.9% at 334mg Kg^{-1}. These changes resulted in the proportion of total PUFAs increasing 4.9% and 3.8% at 167mg Kg^{-1} and 334mg Kg^{-1} respectively while a 1.8% decrease at 834mg Kg^{-1}. The unsaturate: saturate ratio decreased 12.5% and

7.5% at 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels whilst increased 7.5% at the highest treatment level. In the case of the polyunsaturate: saturate ratios increased 12.0% at both 167mg Kg^{-1} and 334mg Kg^{-1} and 3.9% at 834mg Kg^{-1}. Similarly, the *n-6: n-3* ratios also were higher than their baseline level with increases of 9.9%, 3.3% and 2.5% respectively, across the concentration levels.

Significant differences in individual saturates were restricted to 14:0 which decreased 25.6% ($P<0.05$) and 22.8% ($P<0.01$) at the 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels while the monoenoic acids 18:1*n-9* and 18:1*n-7* decreased 8.2% ($P<0.01$) and 9.4% ($P<0.05$) at 167mg Kg^{-1} while the acid 17:1*n-7* decreased 53.8% ($P<0.01$) at 334mg Kg^{-1} and 35.3% ($P<0.001$) at 834mg Kg^{-1}. In the case of individual *n-6* PUFAs, significant changes were restricted to 20:4*n-6* at 167mg Kg^{-1}, which increased 9.7% ($P<0.05$) whilst at 334mg Kg^{-1} there no significant change. At 834mg Kg^{-1}, 18:2*n-6*, 18:3*n-6* and 20:3*n-6* decreased 17.3%, 10.3% and 18.0% at $P<0.05$ significance level. In the case of significant changes in individual *n-3* PUFAs, 22:3*n-3* decreased from 1.09±0.04 to below trace levels ($P<0.001$) at both 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels while 22:6*n-3* increased 12.9% ($P<0.05$) at 334mg Kg^{-1}. The remaining *n-3* PUFAs underwent non-significant changes across the concentration levels.

6.3.1.7. Neonatal Heart Tissue

The effect of the supplementation of oregano volatile oil, as described in the Materials and Methods (Section 6.2.), upon the fatty acid composition of the CE, TAG, FFA and PL fractions from neonatal heart tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6:n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 29 to Appendix 32.

The principal FAs in the CE fraction were the saturates 14:0, 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and tetrænoic 20:4*n-6* *n-6* PUFAs and the 22:6*n-3* hexænoic *n-3* PUFA, Appendix 29. The proportion of total saturates decreased 23.5%, 23.9% and 15.8% across the treatment levels whilst the proportion of total monounsaturates increased 0.8%, 2.5% and 47.5%. The proportion of total *n-6* PUFAs increased 22.2% at 167mg Kg^{-1} while

decreased 26.7% and 4.2% at 334mg Kg^{-1} and 834mg Kg^{-1} concentration levels. In the case of the proportion of total *n-3* PUFAs, there was a 9.2% increase at 167mg Kg^{-1} and decreases of 12.2% and 82.5% at 334mg Kg^{-1} and 834mg Kg^{-1}. The effect of these changes resulted in a 19.4% increase at 167mg Kg^{-1} and decreases of 23.6% and 21.4% at 334mg Kg^{-1} and 834mg Kg^{-1} in the proportion of total PUFAs. The unsaturate: saturate ratios increased 32.7%, 34.6% and 74.6% across the concentration levels while the polyunsaturate: saturate level at 167mg Kg^{-1} increased 57.1%, remained at the baseline level at 334mg Kg^{-1} but decreased 7.1% at 834mg Kg^{-1}. The effect of the oregano treatment levels upon the *n-6:n-3* ratios were cause a 12.1% increases at 167mg Kg^{-1} while at 334mg Kg^{-1} and 834mg Kg^{-1} concentration levels, decreases of 16.6% and 45.5% respectively.

Significant changes in this fraction were restricted to the saturate 14:0 which decreased 81.5% ($P<0.001$) at 167mg Kg^{-1} and to below trace levels at 834mg Kg^{-1}, the saturate 18:0 which decreased 26.4% ($P<0.01$) and 31.5% ($P<0.01$) at 167mg Kg^{-1} and 834mg Kg^{-1} and 20:0 which decreased from a baseline level 1.79±0.19 to below trace levels across the three concentration levels. The monoenoic acid 16:1*n-7* decreased 39.5% ($P<0.01$) at 167mg Kg^{-1} and 79.3% ($P<0.001$) at 834mg Kg^{-1} while 17:1*n-7* decreased to below trace levels at 334mg Kg^{-1} and 834mg Kg^{-1}. The acids 18:1*n-9* and 18:1*n-7* increased 90.8% ($P<0.001$) and 50.0% ($P<0.01$) at 834mg Kg^{-1}. In the cases of the *n-6* PUFAs, significant changes occurred at 167mg Kg^{-1} with the PUFAs 18:2*n-6*, 18:3*n-6* and 20:4*n-6*. Both 18:2*n-6* and 20:4*n-6* increased 30.6% ($P<0.05$) and 48.5% ($P<0.01$) while 18:3*n-6* decreased to below trace levels ($P<0.001$). At the 334mg Kg^{-1} concentration level, 18:2*n-6* decreased 40.3% ($P<0.01$) while the PUFAs 20:3*n-6* and 22:4*n-6* decreased to below trace levels ($P<0.001$). Significant increases in the represented *n-6* PUFAs was restricted to 20:2*n*-6 which at this treatment level increased 116.7% ($P<0.001$). Similarly, at the 834mg Kg^{-1} concentration level, significant increases due to treatment was restricted to 18:2*n-6* which increased 42.8% ($P<0.05$) while the remaining PUFAs 18:3*n-6*, 20:2*n-6* and 20:3*n-6* decreased 82.3% ($P<0.001$), 76.0% ($P<0.01$) and 65.0% ($P<0.01$) respectively. In the case of individual *n-3* PUFAs, significant changes at 167mg Kg^{-1} occurred with the PUFAs 18:4*n-3* which increased 114.5% ($P<0.01$) and 22:6*n-3* which increased 75.1% ($P<0.05$). At 334mg Kg^{-1}, the PUFAs 18:3*n-3*, 20:5*n-3* and 22:5*n-3* decreased to below trace levels ($P<0.001$) while the PUFAs 18:4*n-3* and 22:6*n-3* increased from the baseline levels 0.62±0.09 and 2.73±0.32 to

3.06±0.41 ($P<0.001$) and 5.04±0.57 respectively. At 834mg Kg^{-1}, the *n-3* PUFAs 18:3*n-3*, 18:4*n-3*, 20:5*n-3* and 22:5*n-3* all decreased to below trace levels.

The principal FAs in the TAG fraction were the saturates 14:0, 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and 20:2*n-6* and tetrænoic 20:4*n-6* *n-6* PUFAs and the hexænoic 22:6*n-3* *n-3* PUFA, Appendix 30. The proportion of total saturates increased 11.4%, 15.1% and 15.1% across the treatment levels whilst the proportion of total monounsaturates decreased 11.0% at 167mg Kg^{-1} and increased 23.4% at 334mg Kg^{-1}. In the case of the proportion of total *n-6* PUFAs, there was a 16.5% increase at 167mg Kg^{-1} and a marginal 2.1% increase at 834mg Kg^{-1} but a 10.4% decrease at 334mg Kg^{-1}. The proportion of total *n-3* PUFAs increased 11.9% and 25.0% at 167mg Kg^{-1} and 334mg Kg^{-1} but decreased 33.5% at 834mg Kg^{-1}. These changes in the proportion of total *n-6* and *n-3* PUFAs resulted in the proportion of total PUFAs to increase 15.6% at 167mg Kg^{-1} but to decrease 3.6% and 4.7% across the remaining concentration levels. The unsaturate: saturate ratios varied across the concentration levels, with a 19.1% and 13.3% decrease at 167mg Kg^{-1} and 834mg Kg^{-1} concentration levels while a 7.6% increase occurred at the 334mg Kg^{-1} concentration level. In the case of the polyunsaturate: saturate ratios, there was a marginal 3.6% increase at the 167mg Kg^{-1} concentration level. However, across the remaining treatments, the polyunsaturate: saturate ratios decreased 16.9%. The *n-6:n-3* ratio increased marginally at 167mg Kg^{-1} but decreased 28.1% and 53.7% at 334mg Kg^{-1} and 834mg Kg^{-1} treatment levels.

Significant changes in individual saturates in this fraction were mainly restricted to changes at the 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels. The saturate 12:0 decreased to below trace levels ($P<0.001$) across the three treatment levels whilst 14:0 decreased from the baseline level 1.59±0.15 to below trace levels ($P<0.001$). The saturate 17:0 decreased 24.6% ($P<0.05$) at 167mg Kg^{-1} while at 334mg Kg^{-1}, increased 59.0% ($P<0.05$). The saturate 18:0 decreased 35.3% ($P<0.001$) and 16.1% ($P<0.05$) at 167mg Kg^{-1} and 334mg Kg^{-1}. Significant changes occurred in the monoenoic acids 16:1*n-7* which increased 53.3% ($P<0.05$) at 834mg Kg^{-1}, 18:1*n-9* which increased 32.9% ($P<0.05$) at 334mg Kg^{-1}, 20:1*n-9* which increased 34.6% ($P<0.05$) at 334mg Kg^{-1} and 22:1*n-9* which decreased 25.0% ($P<0.05$) at 167mg Kg^{-1}. Significant changes occurred in the *n-6* PUFAs 18:2*n-6* which increased 25.6% ($P<0.05$) at 167mg Kg^{-1}, 20:2*n-6* which decreased 44.6%

(P<0.001) at 167mg Kg^{-1}, 20:3*n-6* which increased 108.9% (P<0.01) at 167mg Kg^{-1}, 20:4*n-6* which decreased 47.1% (P<0.001) at 334mg Kg^{-1} but increased 28.2% (P<0.001) at 834mg Kg^{-1} and 22:4*n-6* which decreased from the baseline level 0.51±0.13 to below trace levels (P<0.001) and increased to 1.40±0.16 (P<0.001) at 834mg Kg^{-1}. Significant changes occurred in the *n-3* PUFAs 18:3*n-3* which decreased to below trace levels, 18:4*n-3* which at 167mg Kg^{-1} and 834mg Kg^{-1} decreased to below trace levels but increased 86.3% (P<0.001) at 334mg Kg^{-1}, 20:5*n-3* which decreased to below trace levels (P<0.001) at 334mg Kg^{-1} and 834mg Kg^{-1} concentration levels and 22:5*n-3* which increased 79.5% (P<0.001) at 167mg Kg^{-1} and decreased 31.5% (P<0.01) at 834mg Kg^{-1}.

The principal FAs in the FFA fraction were the saturates 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and 20:2*n-6*, the trienoic 20:3*n-6* and tetrænoic 20:4*n-6* and 22:4*n-6* *n-6* PUFAs and the pentænoic 20:5*n*-3 and 22:5*n-3* and hexænoic *22*:6*n-3* *n-3* PUFAs, Appendix 31. The proportion of total saturates marginally increased 7.8% at 167mg Kg^{-1} but decreased 10.6% and 9.7% at 334mg Kg^{-1} and 834mg Kg^{-1} concentration levels whilst the proportion of total monounsaturates decreased 1.0% and 1.6% at 167mg Kg^{-1} and 334mg Kg^{-1} treatment levels and increased 6.3% at 834mg Kg^{-1}. The proportion of total *n-6* PUFAs increased 4.0%, 3.6% and 6.0% across the concentration levels whereas the proportion of total *n-3* PUFAs increased 14.5%, 7.4% and 5.8%. These changes resulted in marginal increases of 6.5%, 4.5% and 5.9% at 167mg Kg^{-1}, 334mg Kg^{-1} and 834mg Kg^{-1} concentration levels in the proportion of total PUFAs. The unsaturate: saturate ratio decreased 9.0% at 167mg Kg^{-1}, however, increased 10.5% and 17.9% across the remaining concentration levels. A similar trend occurred in the polyunsaturate: saturate ratios, with a 1.2% decrease at 167mg Kg^{-1} while increases of 17.1% and 17.7% occurred at 334mg Kg^{-1} and 834mg Kg^{-1} concentration levels. The *n-6:n-3* ratios tended to decrease, with decreases of 9.3% and 3.7% at 167mg Kg^{-1} and 334mg Kg^{-1} respectively.

Significant changes in individual saturates in this fraction included 12:0 which decreased to below trace levels at 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels, 14:0 which decreased 44.3% (P<0.05) and 57.1% (P<0.01) at 334mg Kg^{-1} and 834mg Kg^{-1} and 18:0 which decreased 12.3% (P<0.01), 11.5% (P<0.01) and 6.7% (P<0.01) across the concentration levels. Significant changes occurred in the

monoenoic acids 14:1*n-5* which decreased to below trace levels ($P<0.01$), 16:1*n-7* which decreased 37.9% ($P<0.05$) and 45.2% ($P<0.05$) at 167mg Kg^{-1} and 334mg Kg^{-1}, 18:1*n-9* which increased 8.7% ($P<0.01$) at 834mg Kg^{-1}, 18:1*n-7* which increased 10.1% ($P<0.05$) at 167mg Kg^{-1} and 22:1*n-9* which increased 44.4% ($P<0.01$) at the 167mg Kg^{-1} concentration level. Significant changes in individual *n-6* PUFAs were restricted to 18:3*n-6* which increased 23.4% ($P<0.01$) at 834mg Kg^{-1}, 20:2*n-6* which decreased 55.7% ($P<0.001$) and 44.5% ($P<0.001$) at 167mg Kg^{-1} and 845mg Kg^{-1}, 20:3*n-6* which increased 17.9% ($P<0.01$), 19.1% ($P<0.05$) and 16.1% ($P<0.05$) respectively and 22:4*n-6* which increased 27.1% ($P<0.05$) at 167mg Kg^{-1}. Significant changes in individual *n-3* PUFAs included 18:3*n-3* which decreasing 73.3% ($P<0.05$) at 167mg Kg^{-1}, 82.1% ($P<0.01$) at 334mg Kg^{-1} and 69.7% ($P<0.001$) at 834mg Kg^{-1}, 20:5*n-3* which increased 36.0% ($P<0.01$) at 834mg Kg^{-1}, 22:3*n-3* which decreased from the baseline level 0.32±0.03 to below trace levels at 167mg Kg^{-1} and 334mg Kg^{-1} ($P<0.001$), 22:5*n-3* which increased 47.2% ($P<0.001$) and 28.0% ($P<0.01$) at 167mg Kg^{-1} and 334mg Kg^{-1} and 22:6*n*-3 which increased 37.4% ($P<0.01$) at 167mg Kg^{-1}, 35.8% ($P<0.01$) at 334mg Kg^{-1} and 19.4% ($P<0.05$) at 834mg Kg^{-1}.

The principal FAs in the PL fraction included the saturates 16:0 and 18:0; the monounsaturates 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6*, the trienoic 20:3*n-6* and tetrænoic 20:4*n-6* and 22:4*n-6* *n-6* PUFAs and the pentænoic 22:5*n-3* and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 32. The proportion of total saturates decreased 3.8% and 2.1% at 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels whilst increased 1.5% at 834mg Kg^{-1}. The proportion of total monounsaturates decreased 17.2% at 167mg Kg^{-1} and increased 2.2% and 24.7% at 334mg Kg^{-1} and 834mg Kg^{-1} treatment levels.

The proportion of total *n-6* PUFAs increased 18.1% the at the 167mg Kg^{-1} concentration level, however, maintained baseline levels at the remaining concentrations. In the case of the proportion of total *n-3* PUFAs, there were decreases of 61.3% and 25.0% at 167mg Kg^{-1} and 834mg Kg^{-1} treatment levels while an increase of 5.6% occurred at 334mg Kg^{-1}. The effect of these changes resulted in a marginal 2.6% increase at 167mg Kg^{-1} while minor decreases of 0.3% and 4.7% at 334mg Kg^{-1} and 834mg Kg^{-1} in the proportion of total PUFAs. The unsaturate: saturate ratios varied across the treatment levels, with a 15.4% decrease occurring at 167mg Kg^{-1} and increases of 5.1% and 23.1% occurring at 334mg Kg^{-1} and 834mg

Kg^{-1} concentration levels while the polyunsaturate: saturate ratios increased 6.9%, 1.4% and 5.6% across these concentrations. The *n-6:n-3* ratio increased dramatically from the baseline level at 167mg Kg^{-1} whilst at 834mg Kg^{-1} the increase was in the region of 33.4%.

Significant changes in individual saturates was restricted to 17:0 which decreased 15.0% ($P<0.01$) at 334mg Kg^{-1}. Significant changes in monoenoic acids included 14:1*n-5* which decreased to below trace levels ($P<0.001$) across the three concentration levels, 18:1*n-9* which decreased 20.9% ($P<0.01$) and increased 30.7% ($P<0.001$) at 167mg Kg^{-1} and 834mg Kg^{-1} concentration levels, 18:1*n-7* which increased 13.2% ($P<0.05$) at 834mg Kg^{-1} and 22:1*n-9* which decreased 20.0% ($P<0.01$) at 334mg Kg^{-1}. Significant changes which occurred in individual *n-6* PUFAs included 18:2*n-6* which decreased 6.4% ($P<0.05$) at 167mg Kg^{-1}, 18:3*n-6* which decreased 44.0% ($P<0.01$) and 28.0% ($P<0.05$) at the 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels, 20:4*n-6* which increased 33.1% ($P<0.001$) at 167mg Kg^{-1} and 22:4*n-6* which similarly increased 38.6% ($P<0.01$) at 167mg Kg^{-1}. In the case of significant changes which occurred in individual *n-3* PUFAs included 18:4*n-3* which decreased to below trace levels at 834mg Kg^{-1}, 18:4*n-3* which decreased to below trace levels across the concentration levels, 20:5*n-3* which decreased 38.6% ($P<0.05$) at 167mg Kg^{-1}, 22:5*n-3* which decreased 84.7% ($P<0.001$) at 167mg Kg^{-1} and 22:6*n-3* which similarly decreased 55.1% ($P<0.001$) and 21.9% ($P<0.05$) at 167mg Kg^{-1} and 834mg Kg^{-1} concentration levels.

6.3.1.8. Neonatal Liver Tissue

The effect of the supplementation of oregano volatile oil, as described in the Materials and Methods (Section 6.2.), upon the fatty acid composition of the CE, TAG, FFA and PL fraction from neonatal liver tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6:n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 33 to Appendix 36.

The principal FAs in the CE fraction were the saturates 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6*, trienoic 20:3*n-6* and tetrænoic 20:4*n-6* and 22:4*n-6* *n-6* PUFAs, and the pentænoic 20:5*n-3* and

22:5*n-3* and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 33. The proportion of total saturates increased 12.3%, 14.5% and 18.7% while the proportion of total monounsaturates similarly increased 9.0%, 14.4% and 15.5% across the treatment levels. In the case of the proportion of total *n-6* PUFAs, at the 167mg Kg^{-1} and 834mg Kg^{-1} treatment levels, 10.3% and 2.1% decreases occurred while at 334mg Kg^{-1} a 5.4% increase occurred. The proportion of total *n-3* PUFAs increased 6.3% and 4.9% at 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels but decreased 29.7% at the highest treatment level. The effect of these changes resulted in the proportion of total PUFAs decreasing 5.6% and 10.0% at 167mg Kg^{-1} at 834mg Kg^{-1} levels respectively and a 5.3% increase at 334mg Kg^{-1}. The unsaturate: saturate ratios marginally decreased 2.3% at 167mg Kg^{-1} at 834mg Kg^{-1} treatment levels and remained unaffected at 334mg Kg^{-1} whilst the polyunsaturate: saturate ratios decreased 15.4%, 7.8% and 24.2% across the concentration levels. The *n-6:n-3* ratio decreased 15.7% at 167mg Kg^{-1} but increased 0.8% and 40.2% across the remaining treatment levels.

Significant changes in individual saturates in this fraction included 16:0 which increased 14.9% ($P<0.05$) at 167mg Kg^{-1}, 17:0 which increased 17.1% ($P<0.05$), 45.7% ($P<0.01$) and 31.4% ($P<0.001$), 18:0 which increased 26.9% ($P<0.01$) at 834mg Kg^{-1} and 20:0 which decreased to below trace levels at 334mg Kg^{-1} and 834mg Kg^{-1} treatment levels ($P<0.01$). The monoenoic acids which changed significantly included 14:1*n-5* which increased 107.7% ($P<0.01$) at 167mg Kg^{-1}, 17:1*n-7* which increased 36.7% ($P<0.05$) and 70.0% ($P<0.05$) at 334mg Kg^{-1} and 834mg Kg^{-1} concentration levels, 18:1*n-9* which increased 13.9% ($P<0.05$), 18:1*n-7* which decreased 12.8% ($P<0.01$) at 167mg Kg^{-1} and 20:1*n-9* which decreased 47.1% ($P<0.001$) at 167mg Kg^{-1} and to below trace levels at 334mg Kg^{-1}. Similarly, the *n-6* PUFAs which changed significantly included 18:2*n*-6 and 18:3*n-6* which decreased 31.6% ($P<0.001$) and 35.7% ($P<0.001$) at 834mg Kg^{-1}, 20:2*n-6* which increased 36.7% ($P<0.05$) and 126.7% ($P<0.001$) at 167mg Kg^{-1} and 334mg Kg^{-1} respectively and 20:4*n-6* which decreased 16.1% ($P<0.01$) and 34.9% at 167mg Kg^{-1} and 834mg Kg^{-1} concentration levels. Significant changes in individual *n-3* PUFAs occurred for 18:3*n-3* which increased 440.0% ($P<0.001$) and 260.0% ($P<0.001$) at 167mg Kg^{-1} and 334mg Kg^{-1} respectively, 22:3*n-3* which decreased to below trace levels at these concentrations, 22:5*n-3* which increased 15.0% ($P<0.05$) and 11.5% ($P<0.05$) at

167mg Kg^{-1} and decreased 70.3% ($P<0.001$) at 834mg Kg^{-1} and 22:6*n-3* which decreased 28.8% ($P<0.01$) at 834mg Kg^{-1}.

The principal FAs in the TAG fraction, Appendix 34, were as previously reported in the CE fraction. The proportion of the total saturates decreased 6.6% and 0.20% at 167mg Kg^{-1} and 334mg Kg^{-1} while increased 7.3% at 834mg Kg^{-1}. The proportion of total monounsaturates increased 8.1%, 1.1% and 31.8% across the concentration levels. The proportion of the total *n-6* PUFAs increased 21.3%, 0.5% and 6.0% while the proportion of total *n-3* PUFAs increased 28.9% and 19.2% at 167mg Kg^{-1} and 334mg Kg^{-1}. The effect of these changes resulted in the proportion of total PUFAs increasing 24.5%, 7.7% and 4.2% across the concentration levels. The unsaturate: saturate ratios increased 1.7% at 167mg Kg^{-1}, 2.8% at 334mg Kg^{-1} and 25.0% at 834mg Kg^{-1} while the polyunsaturate: saturate ratios increased 32.1% at 167mg Kg^{-1} and 7.1% at 334mg Kg^{-1}. The *n-6:n-3* ratios decreased 5.7% and 15.9% across the first two concentration levels and increased 6.3% at the highest treatment level.

There were significant changes in individual saturates included 12:0 which increased from 0.10±0.02 to 0.19±0.01 ($P<0.05$) at 167mg Kg^{-1}, 14:0 which increased 64.1% ($P<0.05$) at 834mg Kg^{-1}, 17:0 which decreased 27.7% ($P<0.05$) at 334mg Kg^{-1}, 18:0 which increased 36.5% ($P<0.05$) at 834mg Kg^{-1} and 20:0 which increased from 0.06±0.01 to 0.20±0.04 ($P<0.01$) at 334mg Kg^{-1}. Significant changes in monoenoic acids occurred in 16:1*n-7* which increased 51.7% ($P<0.05$) at 167mg Kg^{-1}, 68.1% 73.6% ($P<0.01$) at 334mg Kg^{-1} and 73.6% ($P<0.01$) at 834mg Kg^{-1}, 18:1*n-9* which increased 27.5% ($P<0.001$) at 834mg Kg^{-1} and 18:1*n-7* which increased 41.6% ($P<0.01$) at the highest concentration level. 20:3*n-6* was the only *n-6* PUFA which changed significantly at any treatment level, increasing 92.5% ($P<0.01$) at 834mg Kg^{-1}. In the case of individual *n-3* PUFAs, significant changes occurred in 18:4*n-3* and 22:3*n-3* which both decreased to below trace levels ($P<0.05$) at 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels, 22:5*n-3* which increased 48.2% ($P<0.01$) and 47.1% ($P<0.05$) at 167mg Kg^{-1} and 834mg Kg^{-1} and 22:6*n-3* which increased 43.9% ($P<0.01$) and 32.4% ($P<0.05$) across the two initial concentration levels.

The principal FAs in the FFA fraction, Appendix 35, were as previously reported in the CE fraction. The proportion of total saturates decreased across the

first two concentration levels 7.1% and 1.9% and 1.3% increase at 834mg Kg^{-1} whilst the proportion of total monounsaturates decreased 18.4%, 8.9% and 3.3% at the treatment levels 167mg Kg^{-1}, 334mg Kg^{-1} and 834mg Kg^{-1}. The proportion of total *n-6* PUFAs decreased 2.5% and 9.2% at 167mg Kg^{-1} and 334mg Kg^{-1} while increased 4.5% at 834mg Kg^{-1}. In the case of the proportion of total *n-3* PUFAs increased 24.0% at 167mg Kg^{-1} and 8.1% at 334mg Kg^{-1} and decreased 2.6% at the 834mg Kg^{-1} treatment level. The effect of these changes resulted in the proportion of the total PUFAs increasing 6.7% and 1.8% at 167mg Kg^{-1} and 834mg Kg^{-1} while at 334mg Kg^{-1} decreased 3.5%.

Significant changes occurred in the saturates 12:0 which increased 188% ($P<0.001$) at 167mg Kg^{-1} and decreased 58.8% ($P<0.001$) at 334mg Kg^{-1}, 14:0 which increased 136.5% ($P<0.001$) and decreased 25.4% ($P<0.01$) at 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels and 17:0 which decreased 25.0% ($P<0.001$) and 15.6% ($P<0.05$) at 167mg Kg^{-1} and 334mg Kg^{-1}. The monoenoic acids 16:1*n-7*, 18:1*n-9*, 18:1*n-7*, and 20:1*n-9* all decreased at the 167mg Kg^{-1} treatment level, 18.4% ($P<0.01$), 17.8% ($P<0.01$), 19.4% ($P<0.01$) and 34.5% ($P<0.01$) respectively while at 334mg Kg^{-1} 16:1*n-7* increased 18.4% ($P<0.05$), 18:1*n-9* decreased 12.6% ($P<0.01$) and 20:1*n-9* decreased 24.1% ($P<0.05$). Significant changes in individual *n-6* PUFAs included 18:2*n-6* which decreased 18.2% ($P<0.001$) at 167mg Kg^{-1}, 18:3*n-6* which decreased 22.1% ($P<0.05$), 23.5% ($P<0.05$) and 26.5% ($P<0.05$) across the concentration levels, 20:2*n-6* which decreased 39.1% ($P<0.01$) and 35.9% ($P<0.05$) at 167mg Kg^{-1} and 334mg Kg^{-1} concentration levels and 20:4*n-6* increased 10.6% ($P<0.05$) at 834mg Kg^{-1}. In the case of significant changes in individual *n-3* PUFAs, 18:3*n-3* increased 75.9% ($P<0.05$) at 167mg Kg^{-1}, 18:4*n-3* decreased from a baseline level 0.23±0.06 to below trace levels ($P<0.05$) across the treatment levels, 22:3*n-3* similarly decreased to below trace levels ($P<0.001$) at 334mg Kg^{-1} and 834mg Kg^{-1} treatments, 22:5*n-3* and 22:6*n-3* increased 30.9% ($P<0.01$) and 27.1% ($P<0.05$) at 167mg Kg^{-1}.

The principal saturated and monounsaturates in the PL fraction, Appendix 36, were as previously reported in the CE fraction. The principal *n-6* PUFAs were the dienoic 18:2*n-6* and tetrænoic 20:4*n-6* acids and the principal pentænoic 22:5*n-3* and hexænoic 22:6*n-6* *n-3* PUFAs. The proportion of total saturates decreased 13.2% at 167mg Kg^{-1} and increased 6.7% at 334mg Kg^{-1} while the proportion of total

monounsaturates decreased 2.2% at 167mg Kg^{-1} and increased 18.2% and 14.9% at 334mg Kg^{-1} and 834mg Kg^{-1} concentration levels. The proportion of total *n-6* PUFAs increased 7.3% at 167mg Kg^{-1} and decreased 10.6% and 3.3% across the 334mg Kg^{-1} and 834mg Kg^{-1} treatment levels. Similarly, in the case of the proportion of total *n-3* PUFAs, at 167mg Kg^{-1} an increase of 41.9% occurred and decreases of 5.4% and 7.7% occurred at 334mg Kg^{-1} and 834mg Kg^{-1}. The proportion of total PUFAs increased 18.1% at 167mg Kg^{-1} and decreased 8.9% and 4.5% at 334mg Kg^{-1} and 834mg Kg^{-1} concentrations. The unsaturate: saturate ratios increased 10.5% across the three treatment levels whilst the polyunsaturate: saturate ratio increased 35.8% at 167mg Kg^{-1} but decreased 14.9% and 4.5% across the remaining concentration levels. The *n-6: n-3* ratios decreased 24.8% and 5.5% at 167mg Kg^{-1} and 334mg Kg^{-1} levels and increased 5.1% at 834mg Kg^{-1}.

Significant changes were restricted to the saturates 16:0 which decreased 17.3% ($P<0.001$) and increased 10.3% ($P<0.05$) at 167mg Kg^{-1} and 334mg Kg^{-1} and 20:0 which decreased 66.7% ($P<0.001$) at 167mg Kg^{-1}. The monoenoic acids which changed significantly at the 167mg Kg^{-1} concentration level included 16:1*n-7*, 17:1*n-7*, 18:1*n-9*, 18:1*n-7* and 22:1*n-9* which decreased 32.7% ($P<0.05$), 34.6% ($P<0.05$), 19.2% ($P<0.05$), 10.1% ($P<0.05$) and to below trace levels ($P<0.01$). At the 334mg Kg^{-1} concentration level, significant changes in these monoenoic acids were restricted to 17:1*n-7* which decreased 26.9% ($P<0.05$) and 22:1*n-9* which decreased to below trace levels ($P<0.01$) while at 834mg Kg^{-1}, 17:1*n-7* decreased 57.7% ($P<0.001$). In the case of significant changes in individual *n-6* PUFAs, 18:2*n-6* increased 6.5% ($P<0.05$) at 834mg Kg^{-1}, 20:2*n-6* decreased 26.7% ($P<0.05$) at 334mg Kg^{-1} and 20:4*n-6* increased 6.9% at 167mg Kg^{-1} and decreased 12.6% at 334mg Kg^{-1}. The *n-3* PUFAs 18:4*n-3* and 22:3*n-3* decreased to below trace levels ($P<0.001$) at 167mg Kg^{-1} while 22:5*n-3* and 22:6*n-3* increased 45.2% ($P<0.01$) and 45.3% ($P<0.001$) at this concentration level.

6.3.2. The Effect of the Oral Administration of Clove or Nutmeg Volatile Oils on the Lipid Composition of Pregnant Rats

6.3.2.1. Maternal Adipose Tissue

The effect of the supplementation of clove or nutmeg volatile oil at 50μg g^{-1} body weight volatile oils, as described in the Materials and Methods (Section 6.2.), upon the fatty acid composition of the TAG fraction from maternal adipose tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6:n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 37.

The principal FAs in the TAG fraction were the saturates 14:0, 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* *n-6* PUFA and the tetrænoic 18:4*n-3* (clove), pentænoic 22:5*n-3* (control) and 22:6*n-3* (clove and nutmeg) *n-3* PUFAs, Appendix 37. The proportions of total saturates decreased in the case of both treatments, decreasing 4.6% with clove and 0.7% with nutmeg while the proportion of total monounsaturates decreased 14.2% and 3.7% respectively with these treatments. While the proportion of total *n-6* PUFAs increased 8.1% with clove volatile oil and 3.7% with nutmeg while the proportion of total and *n-3* PUFAs dramatically increased 225% with clove whilst nutmeg caused a 10.5% increase. The proportion of total PUFAs increased 17.4% in the case of clove while nutmeg produced a 4.5% increase. Changes in the unsaturate: saturate ratios included a 3.4% increase with clove and a 3.4% decrease with nutmeg volatile oils. The polyunsaturate: saturate ratios increased 22.5% and 1.80% respectively. The *n-6*: *n-3* PUFA ratios decreased from the baseline level, 66.8% with clove while 6.41% with the nutmeg treatment.

With the treatment of clove volatile oil, significant changes occurred in the saturates 12:0 which decreased 35.7% ($P<0.001$) and 14:0 which decreased by 20.6% ($P<0.05$). The saturate 20:0 increased from below trace levels to 0.19±0.00 ($P<0.001$). No significant changes occurred in individual monoenoic acids with clove volatile oil at this treatment level. In the case of significant changes in *n-6* PUFAs, clove produced an 8.7% increase ($P<0.05$) in the PUFA 18:2*n-6*. Significant changes in individual *n-3* PUFAs included 18:4*n-3* which increased from below trace levels

to 2.52±0.04 (P<0.001), 22:5*n*-5 which decreased 51.0% (P<0.001) and 22:6*n-3* which increased from below trace levels to 1.11±0.13 (P<0.001).

With the treatment of nutmeg volatile oil, significant changes in individual saturates were restricted to 12:0 which decreased 35.7% (P<0.001) while the monoenoic acids 17:1*n-7* and 20:1*n-9* decreased from 0.26±0.01 to below trace levels (P<0.001) 35.2% (P<0.001) respectively whilst 18:1*n-7* increased 9.4% (P<0.01). The *n-6* PUFAs which changed significantly from the control baseline levels with the nutmeg treatment included 18:3*n-6* which decreased 58.3% (P<0.01) and the PUFAs 20:2*n-6* and 20:4*n-6* which decreased to below trace levels (P<0.001). In the case of significant changes in individual *n-3* PUFAs, 20:5*n-3* decreased from a baseline level 0.19±0.01 to below trace levels, 56.7% (P<0.01) whilst 22:6*n-3* increased from trace levels to 0.99±0.11 (P<0.001) with this treatment.

6.3.2.2. Maternal Brain Tissue

The effect of the supplementation of clove or nutmeg volatile oil at 50μg g^{-1} body weight volatile oils, as described in the Materials and Methods (Section 6.2.), upon the fatty acid composition of the PL fractions from maternal brain tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6:n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 38.

The principal FAs in the PL fraction were the saturates 16:0 and 18:0 (control and nutmeg); the monosaturates 18:1*n-9*, 18:1*n-7* and 20:1*n-9*; the dienoic 18:2*n-6* (clove), tetrænoic 20:4*n-6* and 22:4*n-6* (control and nutmeg) *n-6* PUFAs and the pentænoic 20:5*n-3* (clove) and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 38. The proportion of total saturates decreased 33.1% with clove but increased 7.2% with nutmeg while the proportion of total monounsaturates increased 27.6% with clove but decreased 2.5% with nutmeg. The proportion of total *n-6* PUFAs decreased 1.39% with clove and 5.59% with nutmeg. The proportion of total *n-3* PUFAs increased 24.8% with clove but decreased 15.9% with nutmeg. The result of these changes upon the proportion of total PUFAs were a 12.8% increase with clove volatile oil and a 11.9% decrease with nutmeg volatile oil. The unsaturate: saturate

ration increased 91.4% with the clove treatment and decreased 7.14% with the treatment of nutmeg. Similarly, the polyunsaturate: saturate ratio increased 69.1% while decreased 17.3% with clove and nutmeg volatile oils respectively. In the case of the *n-6*: *n-3* ratio, clove treatment resulted in a 20.3% decrease while nutmeg resulted in a 12.7% increase in this ratio value.

With the treatment of clove volatile oil, significant changes occurred with the saturates 14:0 which increased 75.0% ($P<0.001$), 16:0 which increased 30.0% ($P<0.001$) and 17:0 which increased 23.0% ($P<0.01$) respectively while 18:0 decreased from a baseline level 20.17±0.34 to 0.94±0.00 ($P<0.001$). Significant changes in individual monoenoic acids included 16:1*n-7* which increased 35.7% ($P<0.001$), 17:1*n*-7 which decreased from a baseline level 0.27±0.05 to below trace levels ($P<0.01$), 18:1*n-9* which increased 29.8% ($P<0.01$), 18:1*n-7* which increased 25.1% ($P<0.05$) and 20:1*n-9* which increased 27.25% ($P<0.01$). Significant changes in individual *n-6* PUFAs included 18:2*n-6* which increased 45.2% ($P<0.01$), 20:3*n-6* which increased 19.2% ($P<0.05$) and 20:4*n-6* which increased 30.5% ($P<0.001$) while 22:4*n-6* decreased from a baseline level of 3.43±0.09 to below trace levels ($P<0.001$). In the case of the *n-3* PUFAs, significant changes included 18:4*n-3* which increased from below trace levels to 0.11±0.01 ($P<0.05$), 22:3*n-3* which increased 87.5% ($P<0.001$) and 22:6*n-3* which increased 20.9% ($P<0.001$).

With the treatment of nutmeg volatile oil, significant changes in individual saturates included 14:0, 16:0, 17:0 which increased 25.0% ($P<0.01$), 13.1% ($P<0.001$) and 21.1% ($P<0.01$) respectively while 20:0 decreased 38.4% ($P<0.01$). Significant changes in individual monoenoic acids were restricted to 16:1*n-7* which increased 64.3% ($P<0.001$) and 17:1*n-7* which decreased 51.8% ($P<0.01$). In the cases of individual *n-6* PUFAs, significant changes included 20:3*n-6* which decreased 21.3% ($P<0.01$) and 22:4*n-6* which decreased 15.2% ($P<0.05$) while in the case of individual *n-3* PUFAs, 22:3n-3 decreased from a baseline level 0.16±0.04 to below trace levels and 22:6*n-3* decreased 17.4% ($P<0.01$).

6.3.2.3. Maternal Heart Tissue

The effect of the supplementation of clove or nutmeg volatile oil at 50μg g^{-1} body weight volatile oils, as described in the Materials and Methods (Section X.),

upon the fatty acid composition of the TAG, FFA and PL fractions from maternal heart tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6:n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 39 to Appendix 41.

The principal FAs in the TAG fraction were the saturates 14:0, 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-9*; the dienoic 18:2*n-6* and tetrænoic 20:4*n-6* *n-6* PUFAs; the trienoic 18:3*n-3* (clove), the tetrænoic 18:4*n-3* (control and clove) and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 39. The proportion of total saturates decreased 5.4% and 10.6% with clove and nutmeg volatile oil treatments while the proportion of total monounsaturates increased 10.3% with clove and 1.6% with nutmeg treatments. The proportion of total *n-6* PUFAs increased 1.47% with clove and decreased 0.37% with nutmeg while the proportion of total *n*-3 PUFAs decreased 7.24% with clove and 9.66% with nutmeg. The result of the changes upon the proportion of total PUFAs were to cause a 0.66% increase with clove and a 1.00% decrease with nutmeg. The unsaturate: saturate ratio increased 16.5% with clove and 1.94% with nutmeg while the polyunsaturate: saturate ratio increased 4.65% with clove, it remained at baseline levels in the case of nutmeg. The *n-6: n-3* ratio increased by similar amounts (9.81%) with both volatile oil treatments.

With the treatment of clove volatile oil, significant changes in individual saturates was restricted to 16:0 which decreased 10.0% ($P<0.05$) while in the case of monoenoic acids, significant changes occurred in 16:1*n-7* which increased 34.7% ($P<0.05$) and 17:1*n-7* which decreased 33.3% ($P<0.05$). Significant changes in *n-6* PUFAs occurred in 20:2*n-6* which increased 88.2% ($P<0.001$), 20:4*n-6* which decreased 40.4% ($P<0.05$) and 22:4*n-6* which decreased from a baseline level 0.25±0.02 to below trace levels ($P<0.001$) while significant changes in *n-3* PUFAs was restricted to 20:5*n-3* which decreased from the baseline level 0.09±0.00 to below trace levels ($P<0.05$).

With the treatment of nutmeg volatile oil, there were no significant changes in individual saturates or monoenoic acids with this volatile oil treatment. In the case of *n-6* PUFAs, significant changes were restricted to 20:4*n-6* which decreased 37.1% ($P<0.01$) while significant changes occurred in the *n-3* PUFAs 18:3*n-3* which

increased from a baseline level 0.07±0.03 to 1.34±0.07 ($P<0.001$) and 18:4*n-3* and 20:5*n-3* which decreased from baseline levels 1.38±0.08 and 0.09±0.00 respectively to below trace levels and $P<0.001$ and $P<0.05$ levels of significance respectively.

The principal FAs in the FFA fraction were the saturates 16:0 and 18:0; the monounsaturates 18:1*n-9* and 18:1*n-7*; the dienoic acids 18:2*n-6* and 20:2*n-6* (control and nutmeg) and the tetrænoic 20:4*n-6* *n-6* PUFAs and the pentænoic 22:5*n-3* and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 40. The proportion of total saturates decreased 0.59% with clove and increased 8.26% with nutmeg while the proportion of total monounsaturates increased 9.70% and 47.0% with clove and nutmeg treatments respectively. In the case of the proportion of total *n-6* PUFAs, clove and nutmeg treatments resulted in a 2.48% and 4.46% decreases respectively while the proportion of total *n-3* PUFAs remained at baseline levels with clove and decreased 28.2% with the nutmeg treatment. The effect of these changes upon the proportion of total PUFAs were a 2.08% decrease with clove and a 10.2% decrease with nutmeg. The unsaturate: saturate ratios increased 10.0% and 35.0% with clove and nutmeg treatments while the polyunsaturate: saturate ratios decreased 1.28% and 16.7% with these volatile oils. The *n-6*: *n-3* ratio decreased 2.48% with clove and increased 33.1% with nutmeg.

With the treatment of clove volatile oil, there were no significant changes in individual saturates, however, in the case of individual monoenoic acids, significant change was restricted to 16:1*n-7* which increased 69.7% ($P<0.05$). Similarly, in the case of significant changes in *n-6* PUFAs was restricted to 20:2*n-6* which decreased 63.6% ($P<0.001$). Significant changes in individual *n-3* PUFAs were restricted to 22:n-3 which increased from below trace levels to 0.96±0.58 ($P<0.01$) and 22:6*n-3* which increased 11.2% ($P<0.05$).

With the treatment of nutmeg volatile oil, significant change in the saturates was restricted to 18:0 which increased 17.3% ($P<0.05$) while the monoenoic acids 16:1*n-7* which increased 160.1% ($P<0.001$), 17:1*n-7* which decreased from a baseline level 0.13±0.01 to below trace levels ($P<0.001$) and 18:1*n-9* which increased 59.7% ($P<0.01$). Significant changes occurred in the *n-6* PUFAs 20:3*n-6* which decreased 27.7% ($P<0.05$) and 22:4*n-6* which decreased 27.3% ($P<0.05$). In

the case of *n-3* PUFAs, significant changes occurred in 18:3*n-3* which increased from below trace levels to 0.49±0.03 (P<0.001) while 18:4*n-3* and 20:5*n-3* decreased from baseline levels 0.52±0.02 and 0.27±0.03 to below trace levels (P<0.001) and 22:6*n-3* which decreased 27.9% (P<0.01).

The principal FAs in the PL fraction were the saturates 16:0 and 18:0; the monounsaturates 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and the tetrænoic 20:4*n-6* *n-6* PUFAs and the pentænoic 22:5*n-3* and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 41. The proportion of total saturates decreased 0.51% with clove and increased 6.08% with nutmeg while the proportion of the total monounsaturates increased 27.1% and 21.8% with clove and nutmeg volatile oil treatments respectively. The proportion of total *n-6* and PUFAs increased 9.54% with clove and decreased 0.58% with nutmeg while the proportion of total *n-3* PUFAs increased 30.0% with clove and decreased 17.8% with the nutmeg treatment. These changes resulted in the proportion of total PUFAs to increase 16.3% with clove volatile oil and decrease 6.64% with nutmeg volatile oil. The unsaturate: saturate ratios both increased 31.3% and 18.8% respectively with clove and nutmeg treatments while the polyunsaturate: saturate ratio increased 17.3% with clove and decreased 12.0% with nutmeg. The *n-6*: *n-3* ratios both increased 15.6% and 20.8% respectively.

With the treatment of clove volatile oil, significant change in saturates was restricted to 20:0 which increased from below trace levels to 0.16±0.02 (P<0.01) while the monenoic acids 17:1*n-7* and 20:1*n-9* which similarly increased from below trace levels to 0.17±0.02 (P<0.001) and 0.18±0.02 (P<0.001) respectively. In the case of individual *n-6* PUFAs, significant changes occurred in 18:2*n-6* which increased 25.0% (P<0.05), 18:3*n-6* which increased from below trace levels to 0.17±0.04 (P<0.001) and 20:3*n-6* which increased 44.4% (P<0.05). Significant change occurred in the *n-3* PUFAs 18:4*n-3* which increased from below trace levels to 0.16±0.00 (P<0.001), 22:5*n-3* which increased 42.2% (P<0.05) and 22:6*n-3* which increased 27.2% (P<0.05).

With the treatment of nutmeg volatile oil, significant change in the saturates was restricted to 18:0 which increased 12.0% (P<0.001) while significant increases occurred in the monoenoic acids 17:1*n-7* which increased from below trace levels to 0.17±0.02 (P<0.01), 18:1*n*-9 which increased 22.4% (P<0.001), 18:1*n-7* which increased 12.7% (P<0.01) and 20:1*n-9* which increased from below trace levels to

0.15±0.02 (P<0.01). There were no significant changes in *n-6* PUFAs except in 18:3*n-6* which increased from below trace levels to 0.21±0.03 (P<0.001) while the *n-3* PUFAs 18:3n-3 which increased from below trace levels to 0.10±0.01 (P<0.01), 18:4*n-3* which similarly increased from below trace levels to 0.08±0.01 (P<0.05), 22:5*n-3* which decreased 20.2% (P<0.05) and 22:6*n-3* which decreased 19.1% (P<0.01).

6.3.2.4. Maternal Kidney Tissue

The effect of the supplementation of clove or nutmeg volatile oil at 50μg g^{-1} body weight volatile oils, as described in the Materials and Methods (Section 6.2.), upon the fatty acid composition of the TAG, FFA and PL fractions from maternal kidney tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6:n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 42 to Appendix 44.

The principal FAs in the TAG fraction were the saturates 14:0, 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and tetrænoic 20:4*n-6* (clove) *n-6* PUFAs and the trienoic 18:3*n-3* (nutmeg), the tetrænoic 18:4*n-3* (control and clove) and the hexænoic 22:6*n-3* (clove) *n-3* PUFAs, Appendix 42. The proportion of total saturates increased 25.4% with clove and 2.84% with nutmeg while the proportion of total monounsaturates decreased 13.7% and 5.2% with clove and nutmeg volatile oil treatments respectively. In the case of the proportion of total *n-6* PUFAs, clove produced a 33.1% increase while nutmeg produced a 5.06% increase. The proportion of total *n-3* PUFAs increased 32.2% with clove while the nutmeg treatment produced a 17.8% decrease. The effect of these changes upon the proportion of total PUFAs resulted in a 33.5% increase with clove and a 3.17% increase with nutmeg. The unsaturate: saturate ratios decreased with clove and nutmeg 31.5% and 7.87% respectively while the polyunsaturate: saturate ratio increased 6.74% with clove and remained at baseline levels with the nutmeg treatment. The *n-6:n-3* ratios increased 0.11% with clove and 27.7% with nutmeg treatments respectively.

With the treatment of clove volatile oil, significant changes in saturates included 14:0 which increased 111.2% (P<0.01) and 18:0 which increased 133.9%

(P<0.001) and 20:0 which decreased from a baseline level 0.13±0.03 to below trace levels (P<0.001). In the case of monoenoic acids, significant change was restricted to 16:1*n-7* which decreased 42.0% (P<0.01). The *n-6* PUFA 20:2*n-6* decreased from 0.19±0.00 to below trace levels (P<0.001) and 20:4*n-6* increased 90.0% (P<0.001). Significant change in *n-3* PUFAs was restricted to 22:6*n-6* which increased 143.8% (P<0.001).

With the treatment of nutmeg volatile oil, there were no significant changes in individual saturates, however, in the case of monoenoic acids, 16:1*n-7* decreased 24.1% (P<0.05), 18:1*n-7* increased 7.6% (P<0.05) and 20:1*n-9* decreased 64.2% (P<0.01). Significant change in *n-6* PUFAs was restricted to 18:3*n-6* which decreased 22.2% (P<0.01) and 22:4*n-6* which increased from below trace levels to 0.15±0.02 (P<0.01). In the case of *n-3* PUFAs, 18:3*n-3* increased from below trace levels to 1.38±0.07 (P<0.001) and 18:4*n*-3 decreased from a baseline level of 1.69±0.07 to below trace levels (P<0.001).

The principal FAs in the FFA fraction were the saturates 14:0 (clove), 16:0 and 18:0; the monounsaturates 16:1*n*-7 (clove), 18:1*n-9* and 18:1*n-7*; the dienoic acids 18:2*n-6* and 20:2*n-6* (control and nutmeg), the trienoic acid 20:3*n-6* (control) and the tetrænoic 20:4*n-6* (control and nutmeg) *n-6* PUFAs and the trienoic 18:3*n-3* (nutmeg), the tetrænoic 18:4*n*-3 (clove), the pentænoic 20:5*n-3* (control and nutmeg), 22:5*n*-3 (nutmeg) and hexænoic 22:6*n-3* (control and nutmeg) *n-3* PUFAs, Appendix 43. The proportion of the total saturates increased 2.76% and 3.44% respectively with clove and nutmeg while the proportion of total monounsaturates similarly increased, with clove resulting in a 92.6% increase and 23.5% increase with nutmeg. The proportion of *n-6* PUFAs decreased 29.7% with clove and increased 1.87% with nutmeg while the proportion of total *n-3* PUFAs decreased 55.7% and 3.18% with clove and nutmeg treatments respectively. The effect of the changes upon the proportion of total PUFAs produced a 34.1% decrease with clove volatile oil and a 0.97% increase with the nutmeg treatment. The unsaturate: saturate ratio increased 86.4% with clove and 18.2% with nutmeg while the polyunsaturate: saturate ratios decreased 35.6% and 2.26% respectively. The *n-6*: *n-3* ratios increased 58.7% and 5.14% respectively with the clove and nutmeg treatments.

With the treatment of clove volatile oil, significant changes occurred in the saturates 14:0 which increased from 0.18±0.03 to 1.08±0.06 (P<0.001), 16:0 which

increased 50.0% (P<0.001) and 18:0 and 20:0 which decreased 66.6% (P<0.001) and 25.0% (P<0.05) respectively. In the case of the monoenoic acids, significant changes occurred in 16:1*n-7* which increased from a baseline level of 0.40±0.12 to 4.76±0.74 (P<0.001), 17:1*n-7* which increased 78.6% (P<0.01) and 18:1*n-9* which increased 80.4% (P<0.001). Significant changes in *n-6* PUFAs included 18:2*n-6* which increased 50.3% (P<0.001), 20:2*n-6* which decreased from 1.16±0.07 to below trace levels (P<0.001), 20:3*n-6* which decreased 82.4% (P<0.001), 20:4*n-6* which decreased from a baseline level of 20.58±0.40 to 0.81±0.15 (P<0.001) and 22:4*n-6* which decreased from 0.74±0.03 to below trace levels (P<0.001). Similarly, *n-3* PUFAs 18:4*n-3* increased 184.7% (P<0.001), 20:5*n-3* decreased 85.0% (P<0.001), 22:3*n-3* increased from below trace levels to 0.16±0.00 (P<0.001) and 22:6*n-3* decreased 84.1% (P<0.001).

With the treatment of nutmeg volatile oil, there were no significant changes in saturates, however, significant changes in monoenoic acids included 16:1*n-7* which increased 115.0% (P<0.05), 17:1n-7 which decreased from 0.14±0.02 to below trace levels (P<0.001) and 18:1*n-9* which increased 25.3% (P<0.05). Significant changes in individual *n-6* PUFAs were restricted to 18:2*n-6* which increased 21.2% (P<0.01), 18:3*n-6* which decreased from a baseline level of 0.14±0.01 to below trace levels and 20:4*n-6* which decreased 13.1% (P<0.01). Significant changes in individual *n-3* PUFAs included 18:3*n*-3 which increased from below trace levels to 1.18±0.05 (P<0.001), 18:4*n-3* which decreased from 0.72±0.06 to below trace levels (P<0.001), 22:5*n-3* which increased 105.0% (P<0.01) and 22:6*n-3* which decreased 20.8% (P<0.05).

The principal FAs in the PL fraction were the saturates 16:0 and 18:0; the monounsaturates 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and tetrænoic 20:4*n-6* *n-6* PUFAs and the pentænoic 20:5*n-3* (control and clove) and hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 44. The proportion of total saturates decreased 1.49% with clove and increased 8.67% with nutmeg while the proportion of total monounsaturates increased 16.0% with clove and 21.7% with nutmeg. The proportion of total *n-6* PUFAs increased 5.26% and decreased 4.01% respectively with clove and nutmeg treatments while the proportion of *n-3* PUFAs decreased 8.11% and 34.7% respectively. The effect of these changes upon the proportion of total PUFAs were to produce a 2.69% increase with clove and a 9.71% decrease with nutmeg. The

unsaturate: saturate ratios increased 19.2% and 11.5% respectively while the polyunsaturated ratio similarly increased 4.17% in the case of clove volatile oil treatment but decreased 16.7% with the nutmeg treatment. The *n-6*:*n-3* ratios increased 14.5% with clove and 46.9% with nutmeg.

With the treatment of clove volatile oil, significant change in the saturates was restricted to 20:0 which decreased 50.0% ($P<0.05$) while the monoenoic acids 16:1*n-7* and 17:1*n*-7 increased 126.9% ($P<0.05$) and decreased from the baseline level of 0.19±0.00 to below trace levels ($P<0.001$). Significant changes in individual *n-6* PUFAs were restricted to 18:2*n-6* which increased 29.6% ($P<0.05$) and 22:4*n-6* which decreased from the baseline level of 0.37±0.01 to below trace levels ($P<0.001$). There were no significant changes in individual *n-3* PUFAs with the treatment of this volatile oil.

With the treatment of nutmeg volatile oil, the saturate 18:0 increased 10.0% ($P<0.01$) while the monoenoic acids 16:1*n-7* increased 180.7% ($P<0.001$), 18:1*n-9* increased 17.2% ($P<0.05$) and 18:1*n*-7 increased 20.7% ($P<0.01$). Significant change in *n-6* PUFAs was restricted to 18:2*n-6* which increased 15.2% ($P<0.01$) while the *n-3* PUFA 18:3*n-3* increased from below trace levels to 0.23±0.02 ($P<0.001$), 18:4*n-3* decreased from the baseline level 0.21±0.02 to below trace levels ($P<0.001$), 20:5*n-3* decreased 33.6% ($P<0.001$), 22:5*n-3* decreased 28.1% ($P<0.01$) and 22:6*n-3* decreased 37.0% ($P<0.001$).

6.3.2.5. Maternal Liver Tissue

The effect of the supplementation of clove or nutmeg volatile oil at 50μg g^{-1} body weight volatile oils, as described in the Materials and Methods (Section 6.2.), upon the fatty acid composition of the CE, TAG, FFA and PL fraction from maternal liver tissue with the relevant total saturated-, total monounsaturated-, total *n-3* polyunsaturated- and *n-6* polyunsaturated- and total polyunsaturated fatty acids and the total unsaturated: saturated-, total polyunsaturated: saturated- and *n-6*:*n-3* polyunsaturated fatty acid ratio summaries is shown in Appendix 45 to Appendix 48.

The principal FAs in the CE fraction were the saturates 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and the tetrænoic 20:4*n-6* *n-6* PUFAs and the trienoic 18:3*n-3* (nutmeg), the tetrænoic 18:4*n-3* (control and clove), and the pentænoic 22:5*n-3* and the hexænoic 22:6*n-3* *n-3* PUFAs,

Appendix 45. The proportion of total saturates decreased 12.01% and 14.6% respectively with both clove and nutmeg volatile oils while proportion of total monounsaturates increased 17.8% and 11.0% respectively. In the case of the proportion of total *n-6* PUFAs, clove produced a 2.63% decrease and nutmeg produced a 9.36% increase while the proportion of total *n-3* PUFAs increased 28.4% and 58.3% respectively. The effect of these changes upon the proportion of total PUFAs resulted in a 2.45% and 17.4% increase with clove and nutmeg treatments. The unsaturate: saturate ratios and polyunsaturate: saturate ratios increased 35.1% and 29.7% and 16.1% and 37.6% with clove and nutmeg treatments respectively. The *n-6*:*n-3* ratios decreased 24.1% with clove and 30.8% with nutmeg treatments.

With the treatment of clove volatile oil, significant change in the saturates was restricted to 17:0 which increased from below trace levels to 0.50±0.07 ($P<0.001$) while the monoenoic acid 16:1*n-7* decreased 30.8% ($P<0.05$), 17:1*n-7* and 20:1*n-9* increased from below trace levels to 0.25±0.05 ($P<0.01$) and 0.39±0.14 ($P<0.01$) respectively and 18:1*n-7* increased 34.0% ($P<0.05$). Significant changes in *n-6* PUFAs occurred with 20:2*n-6* which decreased 52.9% ($P<0.05$) and 20:3*n-6* which increased from below trace levels to 0.27±0.04 ($P<0.05$). Similarly, significant changes in *n-3* PUFAs included 20:5*n-3* which increased from below trace levels to 0.77±0.13 ($P<0.001$) and 22:6*n-3* which increased 46.9% ($P<0.05$).

With the treatment of nutmeg volatile oil, significant change in the saturates was restricted to 17:0 which increased from below trace levels to 0.39±0.05 ($P<0.001$) while the monenoic acid 16:1*n-7* decreased 54.7% ($P<0.01$) and 20:1*n-9* increased from below trace levels to 0.50±0.09 ($P<0.01$). There were no significant changes in *n-6* PUFAs except 18:3*n-6* which increased from below trace levels to 0.33±0.02 ($P<0.01$). However, in the case of *n-3* PUFAs, significant changes occurred in 18:3*n-3* which increased from below trace levels to 1.11±0.12 ($P<0.001$), 18:4*n-3* which decreased from the baseline level of 1.51±0.03 to below trace levels ($P<0.001$) and 22:6*n-3* which increased 77.5% ($P<0.01$).

The principal FAs in the TAG fraction were the saturates 16:0 and 18:0; the monounsaturates 16:1*n-7*, 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and tetrænoic 20:4*n-6* *n-6* PUFAs and the tetrænoic 18:4*n-3* (control and clove), the pentænoic 20:5*n-3* and the hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 46. The proportion of total

saturates increased 19.8% with clove and decreased 4.26% with nutmeg while the proportion of total monounsaturates decreased 0.73% and 49.1% respectively with clove and nutmeg volatile oil treatments. The proportion of total *n-6* PUFAs decreased 21.4% with clove and increased 7.79% with nutmeg while the proportion of total *n-3* PUFAs decreased 36.9% with clove and increased 34.2% with nutmeg. The effect of these changes upon the proportion of total PUFAs were to cause a 24.9% decrease with clove and a 15.4% increase with nutmeg. The unsaturate: saturate ratios decreased with both treatments, with clove producing a 17.1% decrease and nutmeg producing a 46.3% decrease while the polyunsaturate: saturate ratio decreased 37.1% with clove, nutmeg caused a 20.5% increase. The *n-6*: *n-3* ratio increased 24.9% with clove and decreased 17.9% with nutmeg.

With the treatment of clove volatile oil, significant change in the saturates was restricted to 18:0 which increased 138.1% ($P<0.001$) while there were no significant changes in the monoenoic acids. In the case of *n-6* PUFAs, significant changes occurred in 18:2*n-6* which decreased 15.7% ($P<0.05$), 20:2*n-6* which increased 46.4% ($P<0.01$), 20:3*n-6* which increased and 100.0% ($P<0.001$) and 20:4*n-6* which decreased from a baseline level of 2.94±0.88 to below trace levels ($P<0.001$). Significant changes in *n-3* PUFAs were restricted to 18:3*n-3* which decreased from a baseline level of 0.09±0.02 to below trace levels ($P<0.01$) and 22:5*n-3* which decreased 55.4% ($P<0.01$). With the treatment of nutmeg volatile oil, there were no significant changes in the saturates. In the case of the monoenoic acids, significant changes occurred in 16:1*n-7* which decreased 56.5% ($P<0.01$), 17:1*n-7* which decreased from a baseline level of 0.17±0.03 to below trace levels ($P<0.01$) and 18:1*n-9* which decreased 51.2% ($P<0.001$). As in the case of the saturates, there were no significant changes in the *n-6* PUFAs while significant change occurred in the *n-3* PUFAs 18:3*n-3* which increased from a baseline level of 0.09±0.02 to 1.68±0.15 ($P<0.001$) and 18:4*n-3* which decreased from a baseline level 1.65±0.27 to below trace levels ($P<0.001$).

The principal FAs in the FFA fraction were the saturates 16:0 and 18:0; the monounsaturates 16:1*n-7 (clove)*, 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and tetrænoic 20:4*n-6 n-6* PUFAs and the trienoic 18:3*n-6* (nutmeg), the tetrænoic 18:4*n-3* (control and clove), the pentænoic 20:5*n-3* and the hexænoic 22:6*n-3 n-3* PUFAs, Appendix 47. The proportion of total saturates increased 14.2% with clove and

increased 0.94% with nutmeg while the proportion of total monounsaturates increased 2.55% with clove and decreased 20.7% with nutmeg. The proportion of total *n-6* PUFAs decreased 9.29% with clove and 3.83% with nutmeg while the proportion of total *n-3* PUFAs increased 16.8% with clove and 21.9% with nutmeg treatments. The effect of these changes upon the proportion of total PUFAs were to produce a 1.99% decrease with clove and a 3.18% increase with nutmeg. The unsaturate: saturate ratios decreased 9.43% and 22.6% respectively with clove and nutmeg while clove treatment caused a 14.7% decrease and nutmeg a 1.91% increase in the polyunsaturate: saturate ratios. The *n-6*:*n-3* ratios decreased 22.1% and 21.0% respectively with these volatile oil treatments.

With the treatment of clove volatile oil, significant change in the saturates were restricted to 14:0 which decreased 47.6% ($P<0.05$) and 18:0 which increased 125.7% ($P<0.001$). Similarly, significant changes in the monoenoic acids were restricted to 16:1*n-7* which increased 48.4% ($P<0.05$), 17:1*n-7* which increased from below trace levels to 0.13±0.03 ($P<0.001$) and 20:1*n-9* which increased from below trace levels to 0.26±0.03 ($P<0.001$). In the case of *n-6* PUFAs, significant changes occurred in 18:2*n-6* which decreased 26.0% ($P<0.05$) and 20:4*n-6* which increased 56.6% ($P<0.01$). Similarly, significant changes in individual *n-6* PUFAs included 22:3*n-3* which increased from below trace levels to 0.23±0.08 ($P<0.001$) and 22:6*n-3* which increased 27.6% ($P<0.05$).

With the treatment of nutmeg volatile oil, significant change in the saturates was restricted to 17:0 which decreased 25.7% ($P<0.05$) while significant changes in the monoenoic acids included 17:1*n-7* and 20:1*n-9* which both increased from below trace levels to 0.12±0.02 ($P<0.001$) and 0.23±0.02 ($P<0.001$) respectively, 18:1*n-9* which 24.1% ($P<0.05$) and 18:1*n-7* which decreased 20.4% ($P<0.05$). In the case of *n-6* PUFAs, 18:3*n-6* decreased 55.1% ($P<0.01$), 20:2*n-6* decreased 40.7% ($P<0.05$), 20:3*n-6* decreased 47.8% ($P<0.01$) and 20:4*n-6* increased 43.9% ($P<0.01$). Significant changes in individual *n-3* PUFAs were restricted to 18:3*n-3* which increased from below trace levels to 1.16±0.24 ($P<0.001$), 18:4*n-3* which decreased from a baseline level 1.77±0.36 to below trace levels ($P<0.001$) and 22:6*n-3* which increased 37.2% ($P<0.05$).

The principal FAs in the PL fraction were the saturates 16:0 and 18:0; the monounsaturates 18:1*n-9* and 18:1*n-7*; the dienoic 18:2*n-6* and tetrænoic 20:4*n-6 n-6*

PUFAs and the pentænoic 22:5*n-3* and the hexænoic 22:6*n-3* *n-3* PUFAs, Appendix 48. The proportion of total saturates increased 0.45% and 10.4% respectively with clove and nutmeg treatments while the proportion of total monounsaturates increased 8.86% with clove and decreased 3.96% with nutmeg. The proportion of total *n-6* PUFAs decreased 1.05% and 15.4% respectively with clove and nutmeg treatments while the proportion of *n-3* PUFAs increased 3.93% with clove and decreased 2.62% with the nutmeg treatment. The result of these changes upon the proportion of total PUFAs were to produce a 0.97% increase with clove and a 9.71% decrease with nutmeg. The unsaturate: saturate ratio increased 10.0% with clove and decreased 20.0% with nutmeg while the polyunsaturate: saturate ratio increased 0.86% and decreased 18.1% respectively with these treatments. The *n-6*: *n-3* ratios decreased 4.80% and 12.8% respectively with clove and nutmeg volatile oil treatments.

With the treatment of clove volatile oil, there were no significant changes in the saturates, except for 14:0 which decreased from a baseline level of 0.07±0.01 to below trace levels ($P<0.01$). Significant changes in the monoenoic acids were restricted to 17:1*n-7* which increased from below trace levels to 0.13±0.01 ($P<0.01$) and 20:1*n-9* which decreased from 0.07±0.01 to below trace levels ($P<0.05$). Similarly, significant changes in *n-6* PUFAs were restricted to 18:3*n-6* which increased 88.9% ($P<0.01$) and 20:3*n-6* which decreased from a baseline level of 0.81±0.14 to 0.27±0.09 ($P<0.01$). There were no significant changes in individual *n-3* PUFAs.

With the treatment of nutmeg volatile oil, significant changes in the saturates and monoenoic acids were restricted to 14:0 which decreased from a baseline level of 0.07±0.01 to below trace levels ($P<0.01$) and 17:1*n-7* which increased from below trace levels to 0.11±0.01 ($P<0.01$). Similarly, significant changes in the *n-6* PUFAs and *n-3* PUFAs were restricted to 18:2*n-6* which decreased from 31.6% ($P<0.01$), 18:4*n-3* which decreased from 0.14±0.01 to below trace levels ($P<0.001$) and 22:5*n-3* which decreased 35.0% ($P<0.01$).

6.4. Discussion

Several investigations have recorded changes in the fatty acid composition of lipid fractions in rats and mice when plant volatile oils have been included as dietary supplements, with particular reference to the process known as ageing (Pénzes *et al.*,

1986; Deans *et al.*, 1993a; Deans *et al.*, 1993b; Feher et. al., 1993; Deans *et al.*, 1994c; Deans *et al.*, 1994b; Deans *et al.*, 1995; Deans *et al.,* 1996; Youdim, 1997.). A similar search of the literature has revealed only a limited number of studies which have investigated the impact of plant volatile oils upon the fatty acid composition within lipids fractions from pregnant rats when included in the diet during the gestation period or their respective in neonates (Simpson, 1995; Youdim *et al.*, 1995; Dorman and Deans, 1997a; Dorman and Deans, 1997b; Dorman and Deans, 1997c). The vast body of studies have been concerned with the effect the composition of the diet, dietary supplementation with polyunsaturated fatty acids (Tinoco *et al.*, 1978; Alsted and Hoy, 1992; Suarez *et. al.*, 1996; Deckere *et. al.*, 1998) or antioxidants (Pénzes *et. al.*, 1988; Jenkins and Atwal, 1995) have on the major fatty acid composition of tissues. Research into neonatal development and nutrition has centered around the effect of unsaturated fatty acids have on lipid composition with the emphasis on designing formula (Crawford *et. al.*, 1989; Hornstra *et. al.*, 1989; Innis, 1991; Simopoulos, 1991; van Houwelingen *et. al.*, 1996; Jimenez *et. al.*, 1996; Harris *et. al.*, 1998) or the effect of fatty acids on the length of the gestation period (Leaver *et. al.*, 1986).

Those studies carried out with plant volatile oils support the hypothesis that plant volatile oils from aromatic and medicinal plants possess *in vivo* antioxidant activity and potentially offer a novel approach to the protection polyunsaturated fatty acids from free radical-mediated degradation associated with ageing. For example, Deans *et al.* (1994b) observed a noticeable *in vivo* antioxidant effect in both the liver and retina of elderly mice fed a standard laboratory diet supplemented with the volatile oil of *Syzygium aromaticum* (L.) Merr. & Perry. The volatile oil was found to afford considerable protection in the maintenance of PUFA levels within the phospholipid fractions, especially for the highly liable C_{22} long-chain polyunsaturated fatty acid docosahexænoate. Similarly, Deans *et al.* (1993b) demonstrated that a variety of plant volatile oils had a marked effect upon fatty acid distribution by virtually restoring the proportions of the polyunsaturated acids within the phospholipids of livers of ageing mice to those levels observed in young mice. In view of these finding, the *in vivo* antioxidant properties of plant volatile oils were investigated, with particular interest in the lipid composition of tissues from pregnant rats and in the oregano treated dams, their neonatal offspring.

It is clear from the results that an effect upon the composition of the major fatty acids within lipid fractions within a number of tissues was observed in dams receiving a dietary supplementation of plant volatile oils prior to and during the gestation period. Furthermore, an effect upon the major fatty acid composition within the lipid fractions of tissues was observed in neonatal rats born to aforementioned dams treated with oregano volatile oil.

The experimental observations that the adipose tissue was almost entirely composed of triacylglyceride not only conforms with what would be expected for this tissue (Christie, 1981), but is indicative that the adipose tissues were exposed to minimum degradation at the time of the analysis (Leskanich and Noble, 1999). In general, the composition of the major fatty acids within the triacylglyceride fraction of adipose tissue includes a high proportions of saturated fatty acids accompanied by significant amounts of unusual components, usually *trans*-unsaturated components (Christie, 1981). In this study, the principal major fatty acid components of the adipose triacylglyceride fractions of both control and treated dams were observed to be the saturated and monoenoic fatty acids palmitate and oleate, and the essential fatty acid linoleate. α-linolenate and the C_{20} polyunsaturated fatty acid arachidonate and the C_{22} long-chain polyunsaturated fatty acids docosapentænoate and docosahexænoate were observed in minor quantities in dams used in the oregano study. However, in the clove and nutmeg studies, α-linolenate and docosahexænoate were observed at trace levels of in the triacylglycerides of untreated dams.

When the relative concentrations of the major individual fatty acids within the adipose triacylglyceride fractions of the dams receiving clove, nutmeg and oregano volatile oil treatments were compared to those of the untreated dams, it appeared that the treatments appeared to have a marginal effect within these fractions. Comparison of the composition of the fatty acids of the triacylglyceride from those rats receiving oregano to those of the control rat triacylglycerides showed that the levels of linolenate and α-linolenate remained unchanged. However, the levels of the C_{20} polyunsaturated fatty acid arachidonate and the C_{22} long-chain polyunsaturated fatty acids docosapentænoate and docosahexænoate decreased. A similar trend was observed with nutmeg volatile oil, e.g. linolenate and α-linolenate levels were unaffected but the levels of arachidonate and docosapentænoate were observed to decrease. Docosahexænoic acid was the exception, its relative composition increased.

Clove treatment had no affect upon α-linolenate and arachidonate levels but linolenate levels were observed to increase as were the levels of docosahexænoic acid. In general, the treatment of oregano volatile oil did not appear to exert any *in vivo* antioxidant effect in the adipose triacylglycerides as the polyunsaturated fatty acids which are particularly liable to peroxidative degradation were not merely maintained at untreated levels but actually decreased. In the case of the clove and nutmeg treatments, the volatile oils failed to protect the levels of the polyunsaturated fatty acids and again a decrease in their respective levels were observed. What was clear, however, was not only a protective effect upon the C_{22} long-chain polyunsaturated docosahexænoate by both clove and nutmeg treatments but there appeared to be an increase the relative level of this fatty acid in this triacylglyceride fractions.

The tissue of the brain is composed mainly of cholesterol and approximately 60% phoshoplipids, and its fatty acid composition is fairly constant amongst animal species, with the predominant *n-6* and *n-3* acids being arachidonate and docosahexænoate (Crawford *et al.*, 1989). The main bulk of brain development takes place during foetal growth and the first two years of life. Post-natally, the emphasis changes to myelination, i.e. making connections between cells. The most essential period for the requirement of long-chain polyunsaturated fatty acids occurs during foetal growth. Consequently, at the perinatal stage an appropriate supply and accretion of docosahexænoate in the membrane phospholipids of the central nervous system is considered essential for visual acuity and neuronal function (Innis, 1991).

The observation that the brain tissue lipid was mainly composed of phospholipids conforms with the expected for this tissue (Christie, 1981). Furthermore, this would tend to suggest that at the time of analysis, degradation was kept to a minimum, especially important in this tissue as it is very rich in the structurally and functionally important C_{20} and C_{22} polyunsaturated fatty acids, which are highly liable to free radical-mediated degradation. When the relative concentrations of the major individual fatty acids within the brain phospholipid fractions of the dams receiving clove, nutmeg and oregano volatile oil treatments were compared to those of untreated dams, it appeared that the oregano treatment had little effect on the major fatty acids within this fraction, while clove and nutmeg treatments demonstrated an effect in this fraction. Comparison of the composition of

the fatty acids of the phospholipids from the untreated dams with those of the oregano treated dams showed the levels of linolenate, arachidonate, docosapentænoate and docosahexænoate fatty acids were maintained at levels seen in untreated animals while the levels of α-linolenate tended to increase with treatments. Comparison of the fatty acid composition of the phospholipids from the control dams to those receiving clove volatile showed that although α-linolenate remained at trace levels, linoleate levels increased. This treatment was also observed to increase the levels of the important polyunsaturated fatty acids arachidonic and docosahexænoic acids. Nutmeg treatment increased the levels α-linolenate, contrary to the actions of clove volatile oil, and decreased the levels of docosahexænoic acid.

The activity of the oregano volatile oil appears to be in general agreement with the observations published by Simpson (1995), of the effect upon the composition of the major fatty acids within the brain phospholipid fractions of dams fed the volatile oils of *Levisticum officinalis* L. and *Thymus vulgaris* L. while the clove and nutmeg treated dams differed. In this Simpson study, both volatile oils were unable to affect the levels of the essential fatty acids or arachidonic, docosapentænoic or docosahexænoic acids within the brain phospholipids. In fact the level of the C_{20} polyunsaturated fatty acid eicosatrienoic acid was the only fatty acid affected in the phospholipid fraction, a result unique to the *Thymus vulgaris* L. treatment.

In terms of the effect upon the major fatty acid composition of the brain phospholipids of neonates born to dams fed oregano volatile oil, a comparison of the fatty acid composition within the brain phospholipids from control neonates with those receiving treatment showed that this oil had limited activity in this fraction. The total levels of linoleate and α-linolenate remained at control levels within this lipid fraction, as did the levels of arachidonate, docosapentænoate and docosahexænoate. These findings are at odds with those reported by Simpson (1995). Both *Levisticum offinalis* L. and *Thymus vulgaris* L. volatile oil treatments had more encompassing effects upon the composition of the fatty acids observed within neonatal brain phospholipids, particularly with reference to saturates and monoenoic fatty acids palmitate, palmitoleate, stearate and oleate. Furthermore, the levels of arachidonate and docosahexænoate polyunsaturated fatty acids decreased in this phospholipids of neonatal brain tissue.

Comparison of the fatty acid composition of the liver cholesteryl esters from the control dams with those receiving oregano volatile oil treatments shows that this oil had no effect on the levels of linoleate, α-linolenate or arachidonate and that the effect upon the levels of the C_{22} long-chain polyunsaturated fatty acids docosapentænoate and docosahexænoate was to decrease their relative importance in this lipid fraction. A comparison of the fatty acid composition of the liver triacylglycerides from untreated dams rats with those receiving this treatments showed a similar trend as seen in the liver cholesteryl esters, with the exception being the levels of docosapentænoate, which remained unaffected in the treated dams. A similar comparison of the fatty acid composition of the free fatty acids shows no change in the total levels of linoleate, arachidonate or docosapentænoate, however, there was a decrease in level of docosahexænoic acid. In the liver phospholipids, comparisons between the levels of linoleic and α-linolenate acids in the untreated dams with these fatty acids in the treated animals showed that both linoleic and α-linolenate levels increased while levels of arachidonate tended to increase. However the levels of docosapentænoic and docosapentænoic were unaffected in the liver phospholipids of oregano treated dams.

In the clove and nutmeg experiments, a comparison of the fatty acid composition of the liver cholesteryl esters of untreated dams with those receiving these plant volatile oils shows that both clove and nutmeg volatile oils had no effect upon the levels of either linoleic or arachidonic acids, common with thyme treatment, α-linoleate levels, with respect to the clove volatile oil. Unlike the oregano treatments, clove and nutmeg had no effect upon the levels docosapentænoate and increased the levels of docosahexænoic acid within the liver cholesteryl ester fractions. A comparison of the fatty acid composition of the liver triacylglycerides from untreated dams with those receiving clove and nutmeg treatments showed these oils had quite different effects within the liver triacylglycerides. The levels of linoleic, α-linolenic, arachidonic and docosapentænoic acid levels all decreased but docosahexænoic acid remained unaffected. Nutmeg volatile oil had no effect on the composition of these fatty acids in the triacylglyceride fraction except for the level of arachidonic acid, which was observed to increase. In the free fatty acid fractions of the liver from untreated dams with those receiving the treatments shows that these oils had similar effects upon the major fatty acids within this fraction, except for the

essential fatty acids. Clove volatile oil reduced the total levels of linoleic acid but had no effect upon α-linolenic acid levels; nutmeg volatile treatment had no effect upon the level of linoleate and cuased the level of α-linolenate to increase. The effect upon the levels of arachidonic and docosahexænoic acid was to increase their compositional levels within this lipid fraction. In the liver phospholipids, the effects of the treatments upon the composition of the fatty acids of this fraction were limited with clove volatile having no effect and nutmeg treatment producing a reduction in the levels of linoleic and docosapentænoic acids.

The effect of the dietary administration of *Levisticum offinalis* L. and *Thymus vulgaris* L. volatile oils upon the composition of fatty acids in liver phospholipids from dams has been published (Simpson, 1993). In the case of *Thymus vulgaris* L., the effect upon long-chain polyunsaturated fatty acids were similar to these findings, with no significant changes in the total levels of linoleate/α-linolenate acids or arachidonate, docosapentænoate and docosahexænoate (Simpson, 1993). Similarly, the findings are in broad agreement with the finds of Deans *et. al.* (1993) who reported that apart from docosahexænoic acid and a concomitant small effect upon total polyunsaturated fatty acid levels, dietary supplementation with plant volatile oils [almond, clove, nutmeg, pepper and thyme] did not produce an effect upon the fatty acid composition of liver phospholipids from young mice.

Deans *et. al.* (1993) proposed that as the phospholipids being the predominant component in the liver lipid and the major carrier of long-chain polyunsaturated fatty acids, especially the C_{20} and C_{22} polyunsaturates (Strickland, 1973), one would expect these observations in the phospholipid fractions to reflect the long-chain polyunsaturated fatty acid compositional changes in the liver as a whole. As shown in this study, the treatments did not affect the fatty acid composition of the lipid fractions of dams in a similar fashion observed in the liver phospholipid fractions. These results suggest that it is important to analyse the other lipid fractions in the liver as changes may not be restricted to the phospholipid fraction.

6.5. Conclusions

Dietary supplementation with unsaturated polyunsaturated fatty acids have been shown to have an effect upon the composition of major polyunsaturated fatty acids in a variety of tissues, with beneficial outcomes for the management of

diseases (British Nutrition Foundation, 1992). The interaction of *n-6* and *n-3* fatty acids and the implications for supplementation of infant formula with long-chain polyunsaturated fatty acids was recently been reviewed (Craig-Schmidt and Huang, 1998). The dietary administration of substances with known antioxidant abilities (e.g. 2-mercaptoethanol) have been shown to be capable of reversing the reduction in the levels of liver PUFA which occurs naturally in the ageing process (Pénzes *et al.*, 1988). However, in terms of the effect of the inclusion of plant-derived antioxidants in the diet upon the fatty acid composition of maternal and their neonatal offspring is limited in the literature.

Simpson (1995) recorded changes in a variety of fatty acids principally in the brain phospholipid fraction and the cholesteryl ester, triacylglyceride, unestified fatty acid and phospholipid fractions of the liver of dams fed thyme and lovage volatile oils prior to and during gestation and also assessed the effect upon neonates born to the aforementioned rats. This study supports the idea that terpene-rich volatile oils are capable of affect the composition of fatty acids within lipid fractions in differnet tissues in both dams and their neonates. However, as to their ability to afford protection to the levels of medium to long-chain polyunsaturated fatty acids within tissue fractions, it is less clear. The idea that these oils are incapable of protecting C_{20} and C_{22} polyunsaturated fatty acids is due to the observations where the levels of arachidonate, docosapentænoate and docosahexænoate decreased with treatment in comparison to the levels in untreated dams and neonate. Interestingly, the effect of a variety of plant volatile upon the fatty acid composition in the phospholipid fraction in young mice receiving these treatments similarly did not show any protected effect (Deans *et al.*, 1995).

7. Concluding Comments

7.1. Overall Discussion

It is clear from the literature that volatile oils from aromatic and medicinal plants possess a variety of important biological activities, chief amongst these are their antibacterial, antifungal and antioxidant properties (Deans and Waterman, 1993). In this study an attempt was made to investigate these properties with particular emphasis upon their potential application in systems. The chemical composition of a selection of plant volatile oils from different plant families was determined, the antimicrobial and *in vitro* antioxidant activities assessed and the hypothesis that the dietary supplementation of plant volatile oils to dams prior to and during the gestation period would have a protective effect upon the compositional levels of the essential fatty acids linoleic and α-linolenic acids and the medium and long-chain polyunsaturated fatty acids arachidonic, docosapentænoic and docosahexænoic acids in lipid fractions extracted from a variety of tissue was tested. Furthermore, would there be a beneficial effect upon the aforementioned fatty acids in neonatal tissues from rats born to these dams.

The plant volatile oils of black pepper, clove, geranium, lovage (leaf material harvested at 3 and 6 months growth and stem), melissa, monarda, nutmeg and a commercially sourced sample, oregano and thyme were analysed using GC-MS and their major components identified using a combination of the retention time of authentic standards when commercially available (single and mixed) and oils of known composition, comparison of chemical analyses in the literature and tentative identification from mass spectra. From the observations made from these and other analyses, it is clear that plant volatile oils are composed of a complex mixture of a range of low molecular weight compounds. The predominant chemical classes of compound were the terpenes and phenylpropanoids. Of these, the terpenes were by far the most numerous, though in the case of clove volatile oil, the main constitutent (% composition) was observed to be eugenol, a phenylpropanoid. Of the terpenes, the most abundant were the mono- (10-carbon skeleton) and sesqui- (15-carbon skeleton) terpenes. The monoterpenes were either acyclic, monocyclic (based upon the *para*-menthane nucleus) or bicyclic (based principally upon either camphane, pinane,

thujane, carane or fenchane nuclei). They were distinguished further through oxidation or reduction of double bonds and by the addition of oxygen to form alcohols (e.g. geraniol/linalool), ketones (e.g. menthone/*iso*-menthone), aldehydes (e.g. citronellal) and esters (e.g. geranyl acetate/terpinyl acetate). Variation in the spatial arrangement of the compounds was often noted.

The antibacterial activity of the plant volatile oils of black pepper, clove, geranium, melissa, nutmeg, oregano and thyme and a number of their constituent components was assessed against a range of important microorganisms, of Gram-negative and Gram-positive types. These included food spoilage bacteria (e.g. *Alcaligenes faecalis*, *Bacillus subtilis*, *Brocothrix thermosphacta* and *Proteus vulgaris*), human and plant pathogens (e.g. *Enterococcus faecalis* and *Escherichia coli*, *Erwinia carotovora*) and food poisoning organisms (e.g. *Clostridium sporogenes*, *Salmonella pullorum* and *Staphylococcus aureus*). The data presented in Table 24 and Table 25 demonstrate that these oils are effective at inhibiting the growth of the test microorganisms, with the oils of oregano and thyme being particularly active. From the chemical composition of these oils and the screening of the major components (Table 26 to Table 34), it is clear that the activity of the most antiseptic oils resides principally in the presence/relative amounts of specific components, e.g. the phenolic compound thymol. Oils which demonstrated more moderate activities appeared to depend less upon high activity/high content of specific compounds but on the variety of moderately active constituents, e.g. compounds with alcohol, aldehyde or ketone functional moieties, which may depend upon the intrinsic activity of their functional groups or through unknown synergistic reactions. However, possible candidate sites of action may include the cytoplasmic membrane (e.g. action on membrane enzymes/potentials/permeability, the electron transport chain or adenosine triphosphate) or the cytoplasm (e.g. action on ribosomes, nucleic acids, thiol groups or amino acids).

Recently, attempts have been made to identify which functional groups or spatial configurations of oil components are responsible for the biocidal activity observed with plant volatile oils. Reports have suggested that the *cis*- configuration around double bonds confers greater antimicrobial activity than the *trans*-configuration and that the most active functional group is the hydroxyl group in both aliphatic alcohols (e.g. linalool) and phenols (e.g. eugenol/thymol). Others have

observed that in a homologous series of aliphatic compounds, chain length is important in determining antibacterial activity relative to Gram-positive and Gram-negative organisms. Even where there is a great deal known about the activity of functional groups, the mechanism of action of oil components is usually poorly understood.

Despite the lack of understanding of the mode of action underpinning the observations in this study or in the literature, the greatest potential exploitation of plant volatile oils with antibacterial activity is in the food industry, especially as their flavours are generally acceptable and are considered by the public as safe organic alternatives to currently used additives. Application in other fields may be limited due to their physical properties (e.g. volatility) and non-specific action (both non-pathogenic and pathogenic bacteria appear to be affected). However, as starting blocks for the development of new antibiotics, an ever increasingly necessary endeavour with the increase in the recognition of strains which are more resistant to current management methods, or in the understanding of novel mechanisms of action, plant volatile oils have an important role to play.

The antifungal activity of the volatile oils of black pepper, clove, melissa, oregano and thyme was assessed against the mycotoxigenic and spoilage fungi *Aspergillus flavus* and *Aspergillus niger* and the plant pathogen *Fusarium culmorum*. *Aspergillus flavus* and *Aspergillus niger* are mycotoxin producing filamentous fungi which are widely encountered as contaminants of cereal crops and foods. Significantly for human health, they are capable of producing aflatoxins which are known to be hepatocarcinogenic. *Fusarium culmorum* is often found as a contaminant in grains and is a considerable cause of economic lost for wheat farmers. Significantly for human and animal health, this fungus is capable of producing mycotoxins associated with illnesses found in farm animals annd humans.

In this study, all the plant volatile oils demonstrated various degrees of antimycotic activity against these fungi, as seen in Figures 23 to 25. The *Aspergillus* species tended to be the most sensitive fungi at the low concentrations the oils were tested at, with *Fusarium culmorum* being the most resistent. However, all species underwent cir. 80% growth inhibition at 10μL mL^{-1} YES broth. The results for *Fusarium culmorum* are particularly significant, as efforts to control *Fusarium* fungal

infections and prevent or eliminate the presence of its mycotoxins in foods have not met with great success (Food Research Institute Briefings, 1997).

In terms of the site of action, the mechanisms underpinning the observations and the components responsible, it is likely that the same susceptible sites are affected as in the case of bacteria, the responsible components are most probably those implicated for the antibacterial activity of plant volatile oils and for the same reasons, e.g. the spatial configuration around double bonds and the presence of a free hydroxyl group as seen in alcohol-based and phenolic components.

The potential application of these plant volatile oils may be in agriculture and in the food industry. It has been reported that *Ocimum basilicum* volatile oil was more effective against 22 fungal species at concentrations which were lower than the recommended application rates of several commercial fungicides and appeared to be unaffected by variation in temperature, conditions of storage and inoculum density (Personal communication, S. Deans). Furthermore, their use is generally considered to be safe.

The *in vitro* antioxidant activity of plant volatile oils was assessed using a range of *in vitro* antioxidant screening methods, which clearly demonstrated that they possess antioxidant properties. Further screening of individual components revealed a range of antioxidant properties which took into account the functional groups of the components. Furthermore, the oils which were specifically rich in phenolic terpenes were often as effective at retarding the formation of the by-products of free radical-mediated unsaturated fatty acid peroxidation in different media and under peroxidation-facilitating conditions (e.g. with iron sulphate/ABAP) as synthetic and naturally occurring antioxidant compounds currently used for this purpose. A reason for this may be structural. For example, BHT has two tert-butyl groups in the ortho-positions, on either side of its phenol function. These are bulky groups which are known to produce steric hindrance and reduce hydrogen bonding occurring in acqueous solution. However, the bulk tends to reduce the access to the hydroxyl group and therefore its ability to scavenge peroxyl radicals. The phenolic compounds carvacrol and thymol do not experience the same level of hindrance and would therefore be more available to react with peroxyl radicals. In terms of mechanism of action, the activities of the volatile oils most probably lies in the availability of functional hydroxyl groups donating a free hydrogen which then interacts in the free

radical chain reactions involved in the initiation and propagation processes of lipid peroxidation. Thymol has been shown to donate hydrogen, a scavenger of peroxyl radicals, and so a possible method of protecting lipid-rich media would be through scavenging peroxyl radicals.

A clear potential use of plant volatile oils or their components would be in food technology. Antioxidants have been incorporated into processed foods to reduce free radical-mediated degradation of polyunsaturated fatty acids and other ingredients for a number of years. The ability to retard peroxidative damage at the storage of raw materials, the processing and the 'on the shelf' stages would have great significance to this industry. An extension of the quality and shelf-life of foods would be of direct benefit to the consumer. Furthermore, any application requiring the protection of a product from lipid peroxidation could potentially benefit from the use of phenolic plant volatile oils. As to the use of these plant products as *in vivo* antioxidants, it is less clear. The role of free radicals in the etiology of numerous diseases and in the process known as ageing is established. However, whether plant volatile oils would be a useful addition for a clinician in the management of such conditions is uncertain. Furthermore, essential questions would have to be addressed prior to further consideration, e.g. the toxicology, the metabolism (including the absorption, distribution, catabolism and excretion) and availability at relevant cellular concentration levels, the targeting and any potential adverse drug/nutrient interactions.

The possibility that the volatile oils of clove, oregano and nutmeg could act as antioxidant dietary supplements during pregnancy was assessed by supplementing a standard laboratory diet fed to dams prior to and during the gestation period with these oils and determining corresponding changes in the fatty acid composition of different lipid fractions of the dams and, in the case of oregano, their neonates.

7.2. Suggestions for Future Research

The results presented in this study have highlighted the rich composition of plant volatile oils and their potential beneficial biological properties as antibacterial and antifungal agents and as *in vitro* antioxidants. However, further research into certain aspects would be of merit. A number of issues come to mind.

7.2.1. Investigations into Chemical Composition

The chemical analyses of the volatile oils used in this study revealed that the component identification was restricted to those components present above trace levels and those which could be identified with a degree of certainty, as described in Chapter 2. Numerous articles have been published which have identified 100's of components present in plant volatile oils from a range of aromatic and medicinal plant families, principally using GC, GC-MS, ^{1}H-NMR and ^{13}C-NMR spectra and fractionation techniques (Baser *et al.*, 1996; Bicchi *et al.*, 1998). It would be very interesting to identify the components of the oils used in this study using these analytical methods.

7.2.2. Investigations into Antibacterial Properties

The water insolubility of plant volatile oils and components and the criteria and practical techniques available, make investigations into the antibacterial properties of aromatic and medicinal plant-derived products problematic. Factors such as the extraction method, the inocula volume, the composition of the culture medium, the pH, the incubation temperature and the volume of sample used can affect a sample's ability to inhibit a microorganism's growth *in vitro*. Additionally, there is no standardized method used in the literature to express antimicrobial activity.

It would be interesting repeat the antibacterial screening of the plant volatile oils used in this study bearing in mind these issues. A variety of techniques could be used including include methods which rely upon diffusion, e.g. disc (Ajao *et al.*, 1985; Maruzzella and Sicurella, 1960; Mathur and Gonzalez, 1982 and Prasad *et al.*, 1986), and hole-plate (Adesina and Akinwusi, 1984); dilution, e.g. tube or turbidimetric methods (Fournier *et al.*, 1978 and Yousef and Tawil, 1980) and bioautography, e.g. contact (McCallion *et al.*, 1982; Rao *et al.*, 1982 and Wallhäuser, 1969), direct (Homans and Fuchs, 1970; Lund and Lyon, 1975; Peterson and Edgington, 1969 and van der Nat *et al.*, 1982) and immersion (Rios *et al.*, 1987 and Villar *et al.*, 1986) methods. Furthermore, it would be interesting to investigate activity against microorganisms which are of increasing concern, e.g. methicillin-

resistant *Straphylcoccus aureus* (MRSA) and mupirocin-resistant MRSA, as the carriage and dissemination of such microorganisms is potentially a serious issue.

7.2.3. *Investigations into Antifungal Properties*

It is important to ensure that any chemical or mechanical method which is described as fungistatic or fungicidal should be proven to possess these properties and not curative influences on the mycoses. Consequently, it should not be assumed that they possess either of these properties simply because it is used in mycotic therapy as its therapeutic purposes may merely be those of soothing and astringency.

7.2.4. Investigations into Antioxidant Properties

In order to confirm that these plant extracts have real use in the modulation of oxidative damage further detailed investigations must be carried out. Potential markers of interest could include the measurement of DNA damage by determining oxidized bases using HPLC and GC-MS methodologies; the measurements of allantoin and uric acid, vitamins, β-carotene, glutathione and plasma antioxidant levels using HPLC; the measurements of antioxidant enzymes such as catalase, glutathione peroxidases and reductases and SOD, the measurements of prostaglandin $F_2\alpha$, thiol specific antioxidants, protein tyrosines and aromatic hydroxylation levels using HPLC; the measurements of lipid damage e.g. degree of fatty acid unsaturation by GC-MS, the measurement of hydroxynonenal and TBA reactive species levels; the assessment of protein damage and the characterisation of any pro-oxidant activities.

7.2.5. Investigations into Fatty Acid Composition

It would be very interesting to investigate whether plant volatile oils, which have demonstrated *in vitro* antioxidant activities, are capable of protecting the levels of unsaturated fatty acids, particularly the medium and long-chain polyunsaturates, in different lipid fractions in animals which are undergoing increased levels of oxidative stress. Under such circumstances, it is likely that deleterious changes in the major fatty acid composition due to carbon tetrachloride treatment, for example, would be

very susceptible to a protective effect of any antioxidant compound or mixture. Such a model would be very informative in assessing plant volatile oils ant their antioxidative capacity.

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Appendices

Appendix 1. Reference retention times for authentic plant volatile oil components, as determined by gas chromatography using a DB1 column.

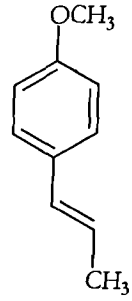
Component	Mixed	Single	Component	Mixed	Single
cis-3-Hexen-1-ol	6.15	6.09	(-)-Fenchone	15.22	15.19
Tricyclene	8.88	8.91	Terpinolene	15.95	16.00
α-Pinene	9.28	9.23	(-)-Thujone	16.11	16.06
Camphene	9.78	9.72	Linalool	16.23	16.17
6-Methyl-hept-5-en-2-one	10.74	10.82	Nonanal	16.26	16.27
(+)-Sabinene	10.74	10.69	(-)-α-Fenchol	16.87	16.82
β-Pinene	10.87	10.82	(+)-Camphor	17.72	17.72
Myrcene	11.53	11.49	Camphor	17.80	17.76
α-Phellandrene	12.06	12.01	(-)-*trans*-Pinocarveol	17.91	17.96
2-Carene	12.12	12.12	(-)-allo-Ocimene	18.02	18.00
δ-3-Carene	12.32	12.36	Menthone	18.27	18.33
Benzyl alcohol	12.46	12.46	Isopulegol	18.30	18.39
α-Terpinene	12.74	12.64	Citronellal	18.58	18.59
p-Cymene	12.75	12.69	Borneol	19.11	19.05
1,8-Cineole	13.05	13.00	Menthol	19.76	19.65
Limonene	13.14	13.10	Terpinen-4-ol	19.77	19.70
R(+)-Limonene	13.24	13.30	α-Terpineol	20.33	20.25
γ-Terpinene	14.48	14.44	(+)-Isomethol	20.26	20.28
cis-Linalool oxide	-	14.71	(-)-Verbenone	20.61	20.52
trans-Linalool oxide	-	15.38	Neral	-	22.54
(+)-Fenchone	15.22	15.19	*cis*-Geraniol	22.40	22.44

(Nerol)

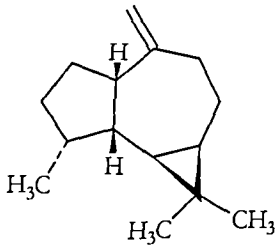
Appendix 1 ***continued.***

Component	Mixed	Single
(-)-Pulegone	-	22.39
(+)-Pulegone	-	22.49
(+)-Carvone	22.43	22.48
(-)-Carvone	22.43	22.48
β-Citronellol	22.46	22.36
Carvacrol methyl ether	-	23.10
trans-Geraniol	23.51	23.59
Geranial	-	23.94
Linalyl acetate	23.74	23.68
trans-Anethole	24.51	24.45
Bornyl acetate	24.91	24.85
Azulene	24.99	24.99
Thymol	25.07	25.00
Carvacrol	25.47	25.40
Eugenol	27.69	27.61
(+)-α-Terpinyl acetate	27.977	28.66
Neryl acetate	28.61	28.66
Geranyl acetate	29.31	29.25
β-Caryophyllene	31.45	31.40
α-Cedrene	31.33	31.39
β-Cedrene	31.59	31.62
(+)-Aromadendrene	32.52	32.46
α-Humulene	33.06	32.84

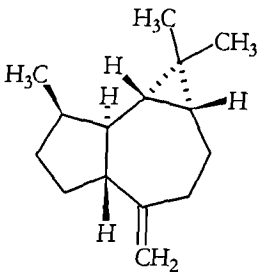
Appendix 2. Structural formulae of compounds in this study.



t-Anethole (**1**)



allo-Aromadendrene (**2**)



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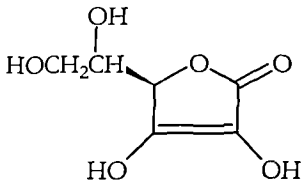
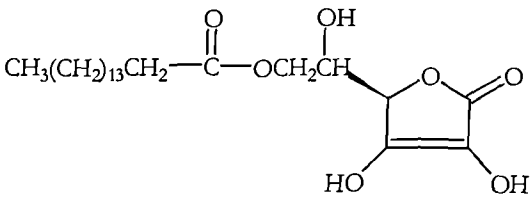
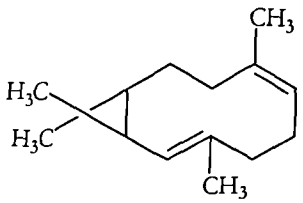


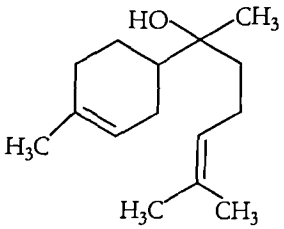
Ascorbic acid (**4**)



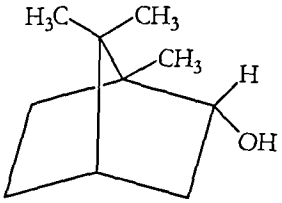
L-Ascorbic acid 6-palmitate (**5**)



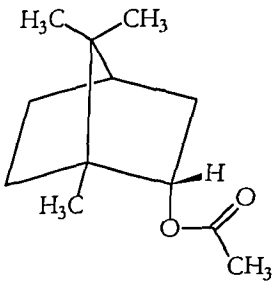
Bicyclogermacrene (**6**)



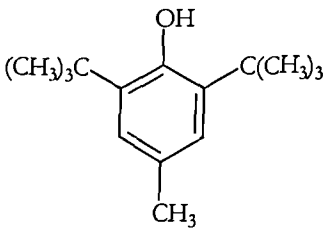
α-Bisabolol (**7**)



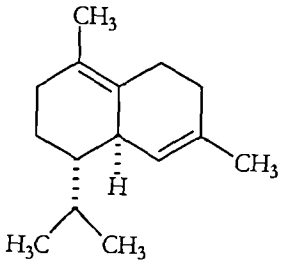
Borneol (**8**)



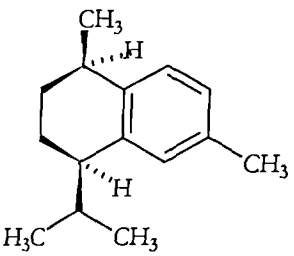
Bornyl acetate (**9**)



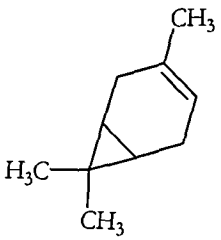
Butylated hydroxy anisole (**10**)



δ-Cadinene (**11**)



1S, *cis*-Calamenene (**12**)



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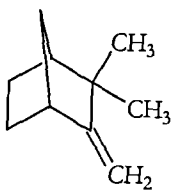
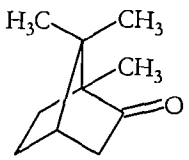
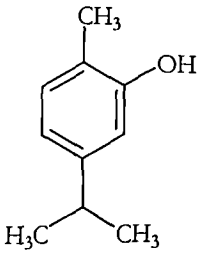


Camphene (**14**)



Camphor (**15**)



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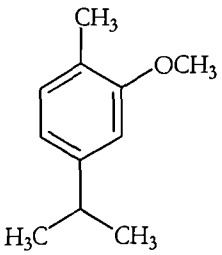
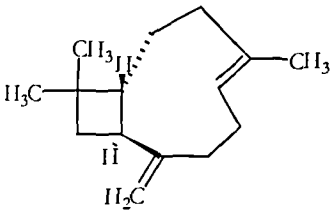
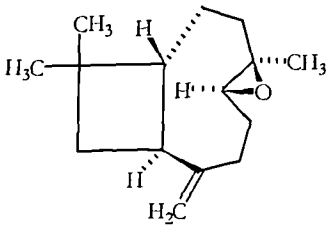


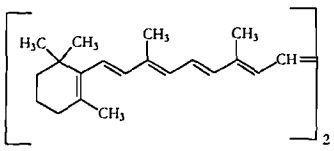
Carvacrol methyl ether (**17**)



β-Caryophyllene (**18**)



Caryophyllene oxide (**19**)



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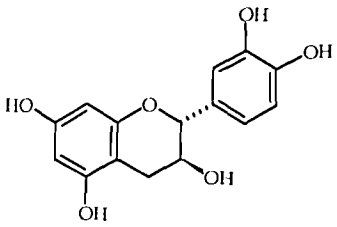
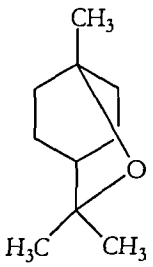
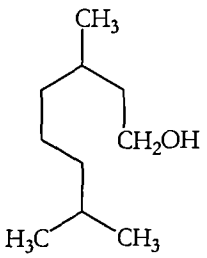


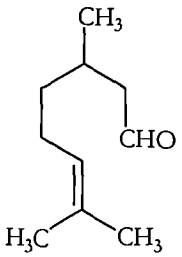
(+)-Catechin hydrate (**21**)



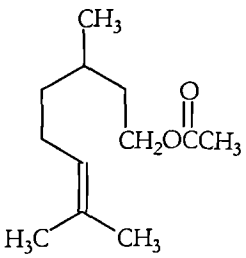
1,8-Cineole (**22**)



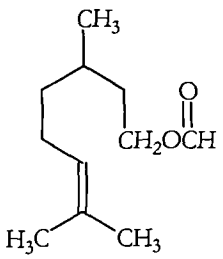
Citronellol (**23**)



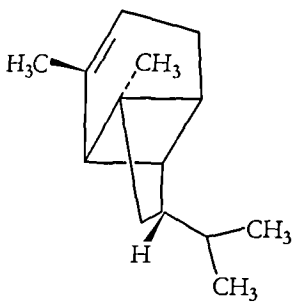
Citronellal (**24**)



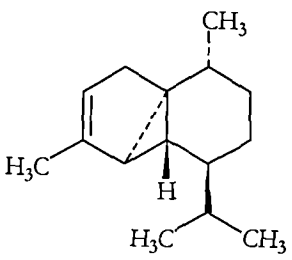
Citronellyl acetate (**25**)



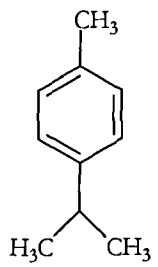
Citronellyl formate (**26**)



α-Copaene (**27**)



α-Cubebene (**28**)



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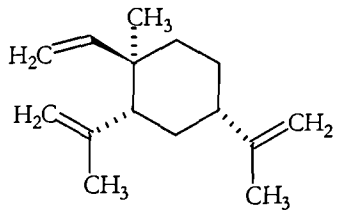
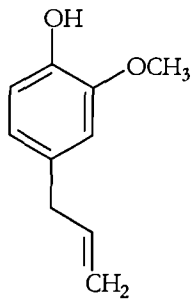


β-Elemene (**30**)



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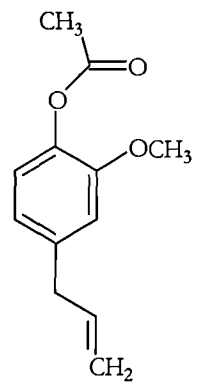

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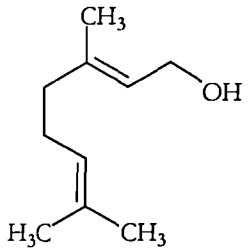
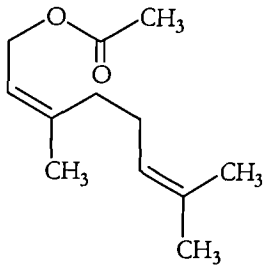
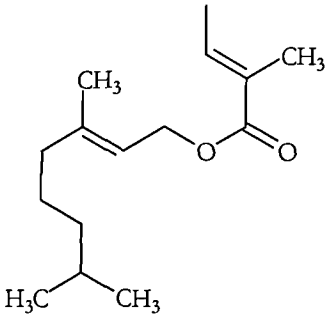


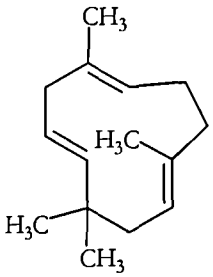
Geraniol (**33**)



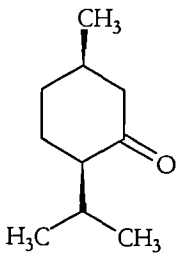
Geranyl acetate (**34**)



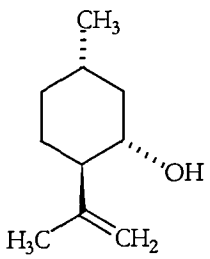
Geranyl tiglate (**35**)



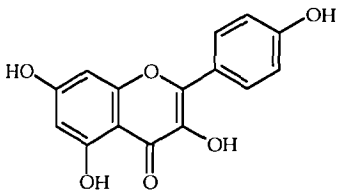
α-Humulene (**36**)



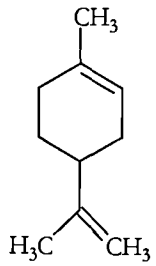
Isomethone (**37**)



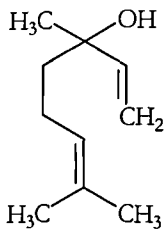
Isopulegol (**38**)



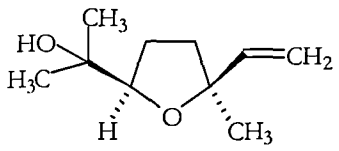
Kaempferol (**40**)



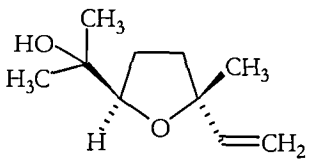
Limonene (**41**)



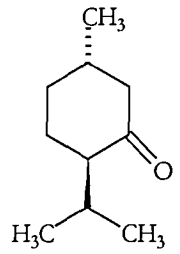
Linalool (**42**)



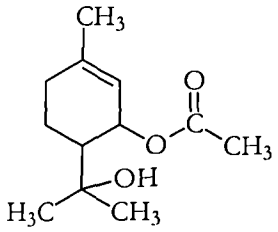
cis-Linalool oxide (**43**)



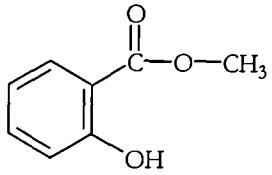
trans-Linalool oxide (**44**)



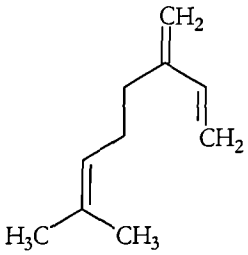
Menthone (**45**)



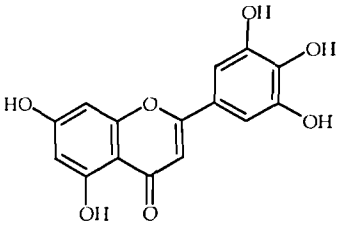
p-Menth-1-en-8-ol, acetate (**46**)



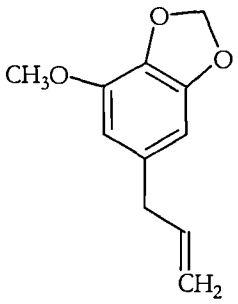
Methyl salicylate (**47**)



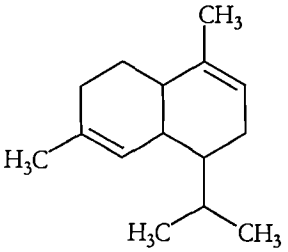
Myrcene (**48**)



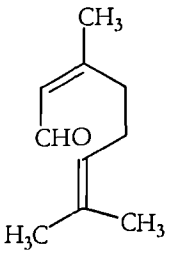
Myricetin (**49**)



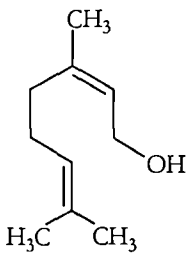
Myristicin (**50**)



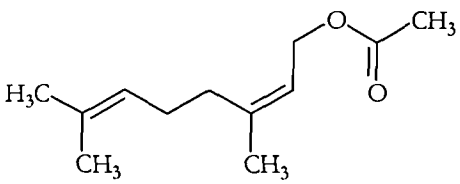
α-Muurolene (**51**)



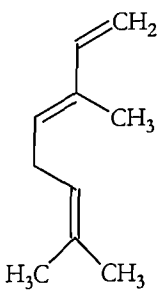
Neral (**52**)



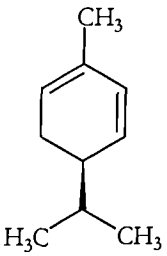
Nerol (**53**)



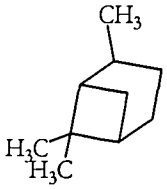
Neryl acetate (**54**)



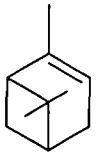
trans-β-Ocimene (**55**)



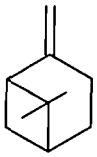
R(-)-α-Phellandrene (**56**)



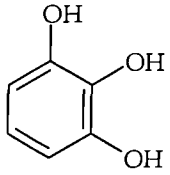
Pinane (**57**)



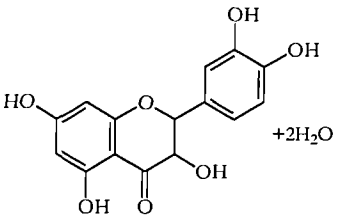
α-Pinene (**58**)



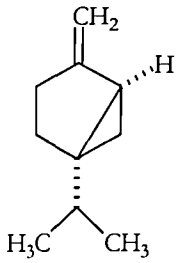
β-Pinene (**59**)



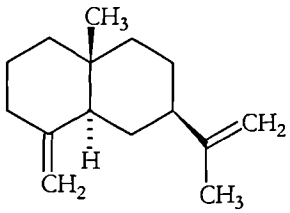
Pyrogallol (**60**)



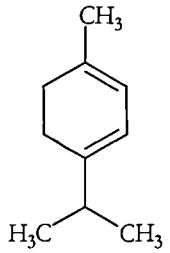
Quercetin dihydrate (**61**)



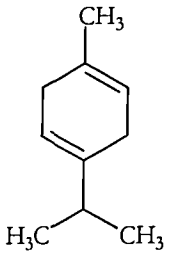
(+)-Sabinene (**62**)



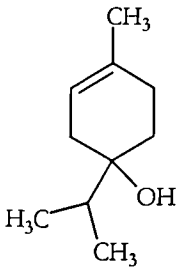
β-Selinene (**63**)



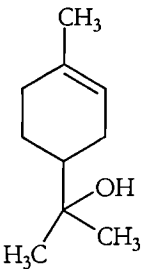
α-Terpinene (**64**)



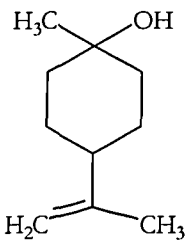
γ-Terpinene (**65**)



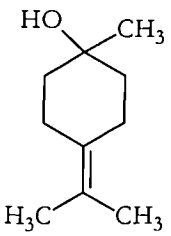
Terpinen-4-ol (**66**)



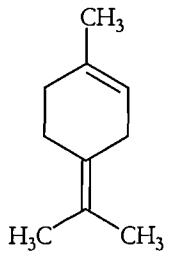
α-Terpineol (**67**)



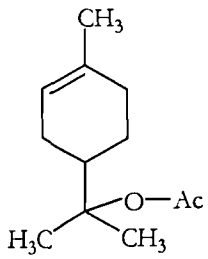
β-Terpineol (**68**)



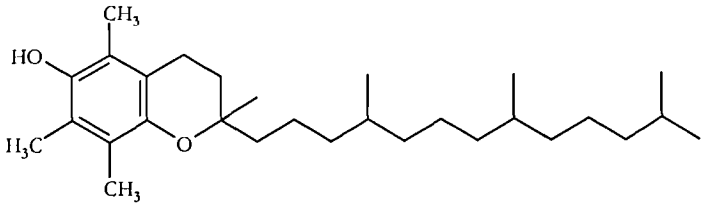
γ-Terpineol (**69**)



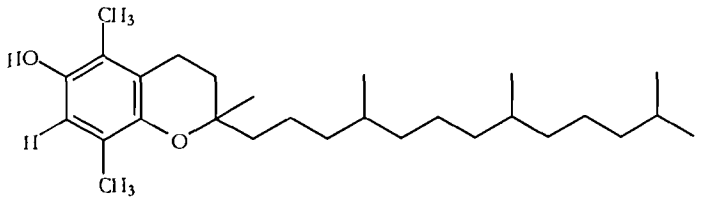
Terpinolene (**70**)



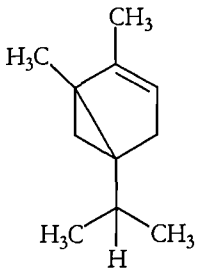
α-Terpinyl Acetate (**71**)



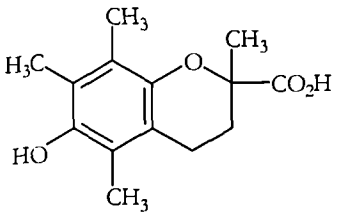
α-Tocopherol (**72**)



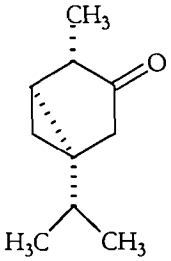
β-Tocopherol (**73**)



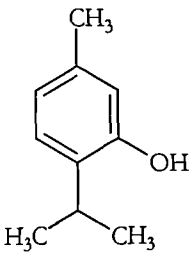
α-Thujene (**74**)



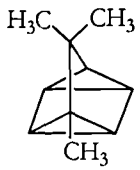
Trolox (**75**)



α-Thujone (**76**)



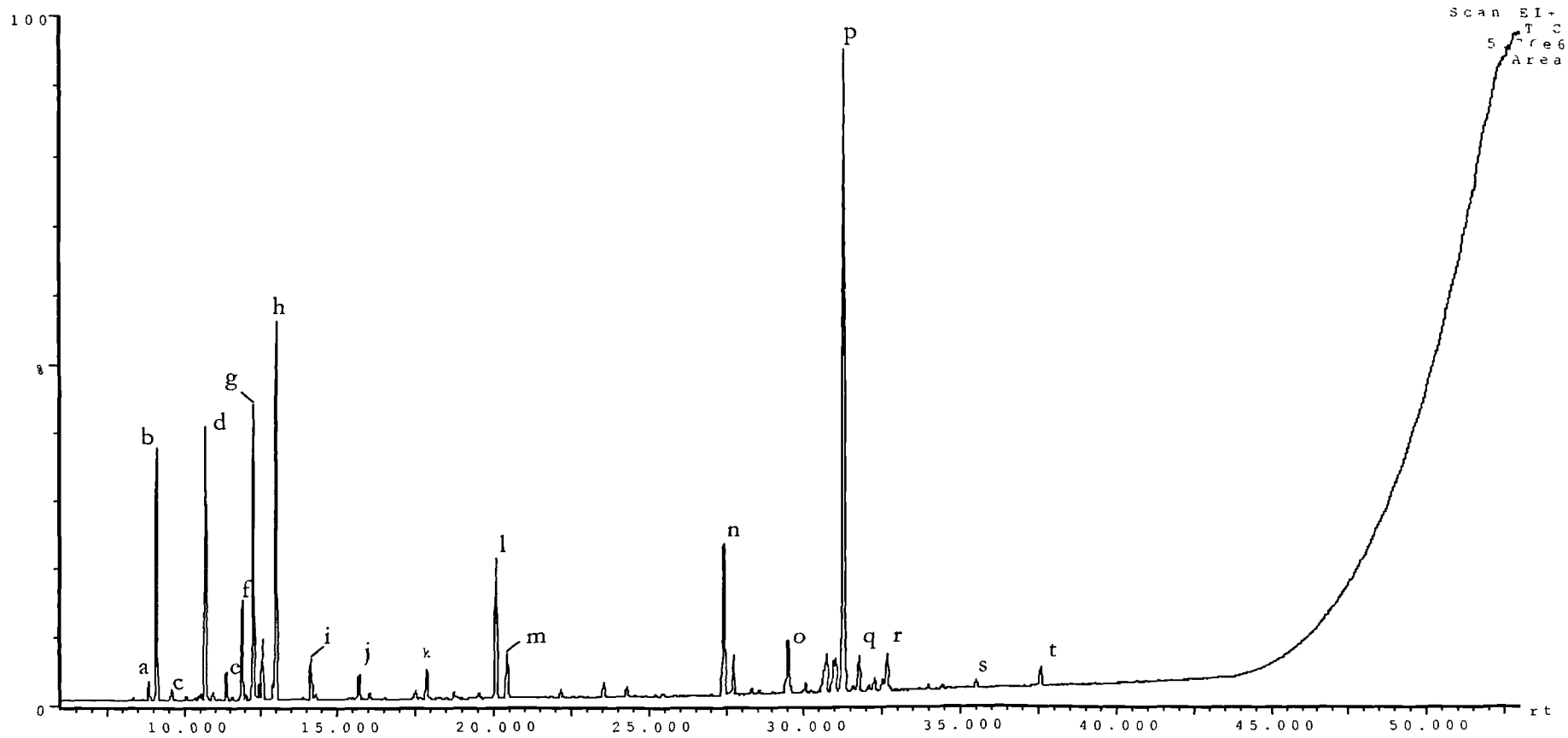
Thymol (**78**)



Tricyclene (**79**)

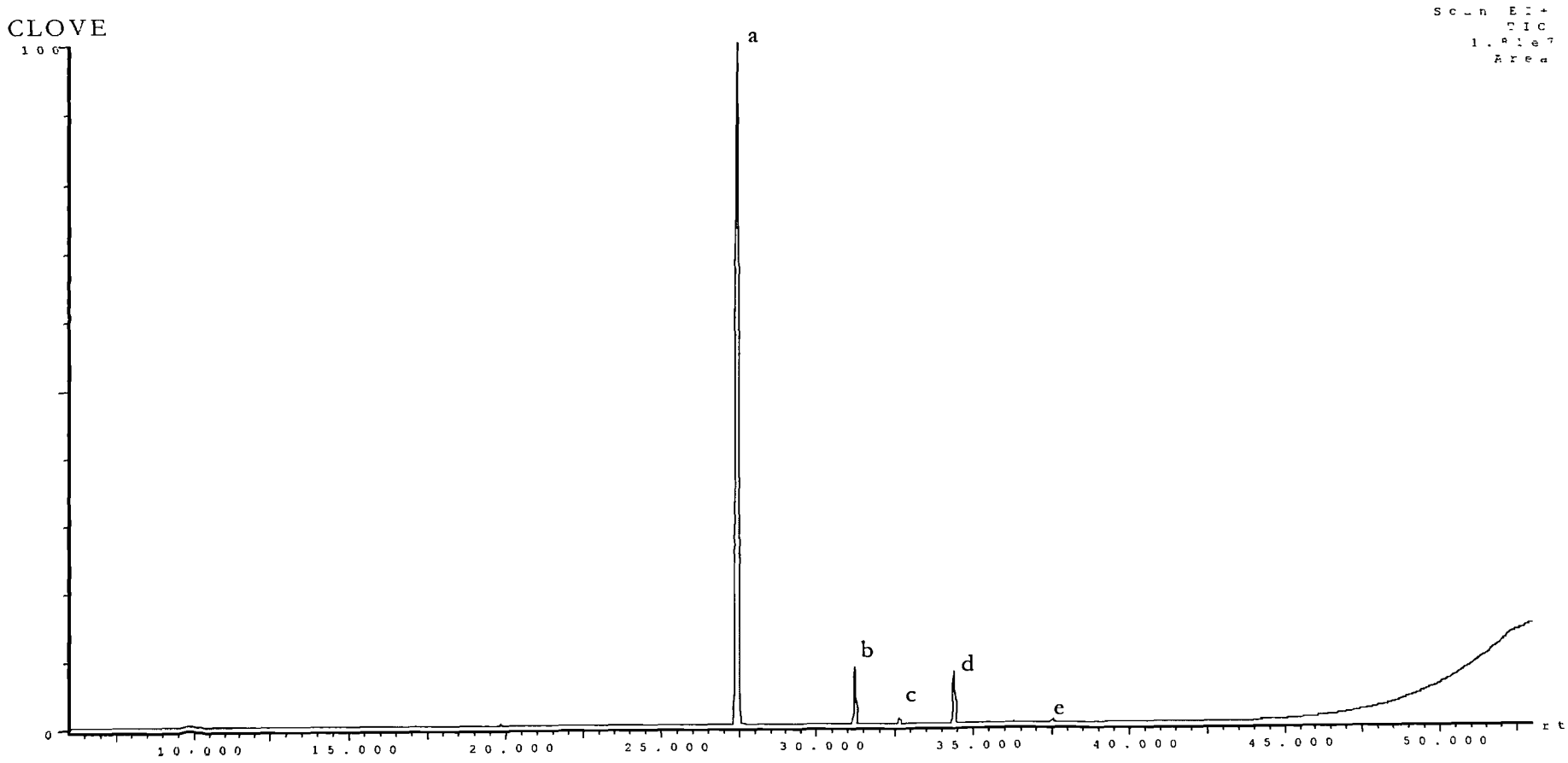
Appendix 3. GC-MS chromatogram of black pepper volatile oil.

BLACK PEPPER



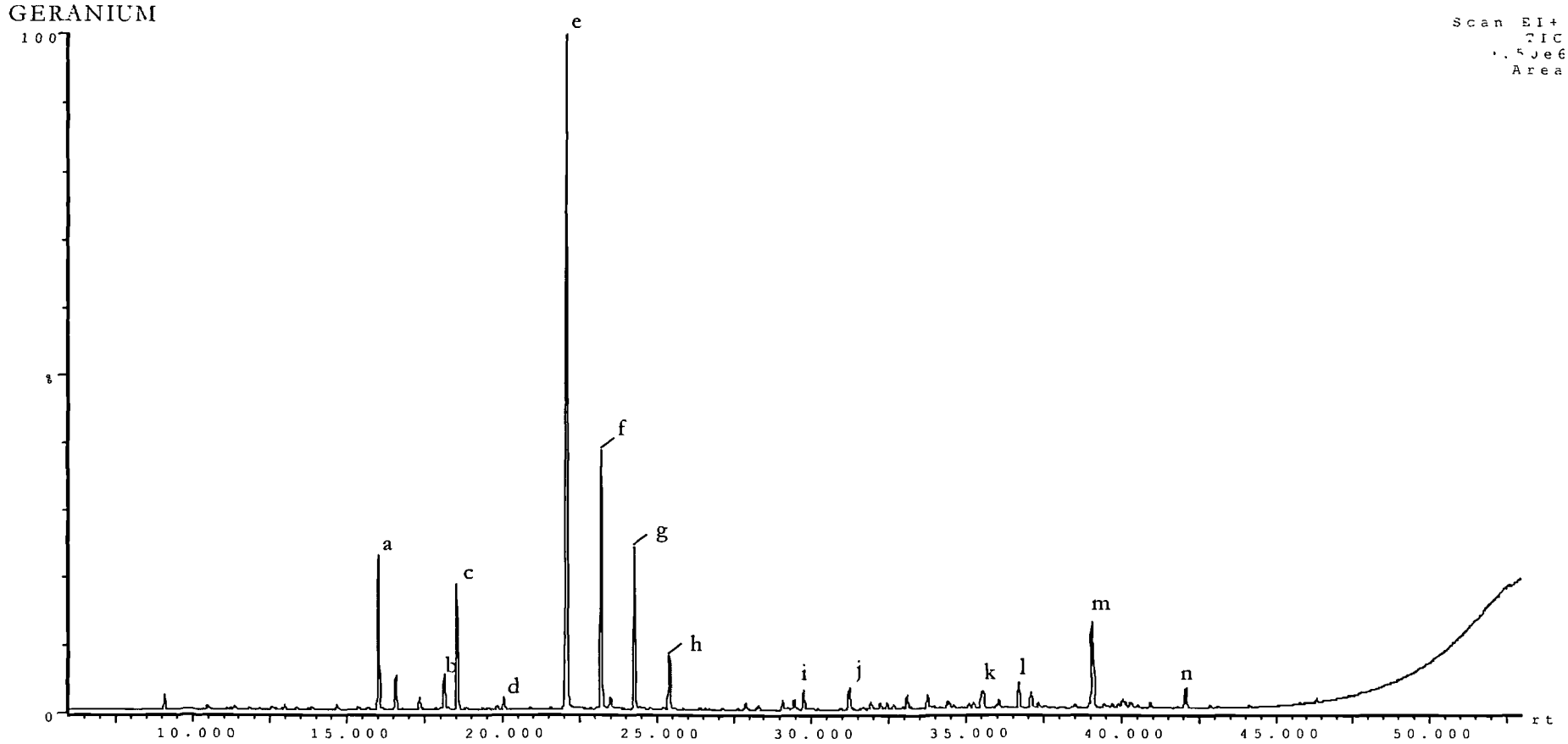
Peak Key: **a**: α-Thujene; **b**: α-Pinene; **c**: Camphene; **d**: β-Pinene; **e**: Myrcene; **f**: R(-)-α-Phellandrene; **g**: δ-3-Carene; **h**: R(+)-Limonene; **i**: Formic acid phenyl methyl ester; **j**: Terpinolene; **k**: Cyclohexanol; **l**: α-Terpineol; **m**: γ-Terpineol; **n**: Eugenyl acetate; **o**: α-Copaene; **p**: β-Caryophyllene; **q**: unidentified; **r**: α-Humulene; **s**: β-Cedrene **t**: β-Citronellol.

Appendix 4. GC-MS chromatogram of clove volatile oil.



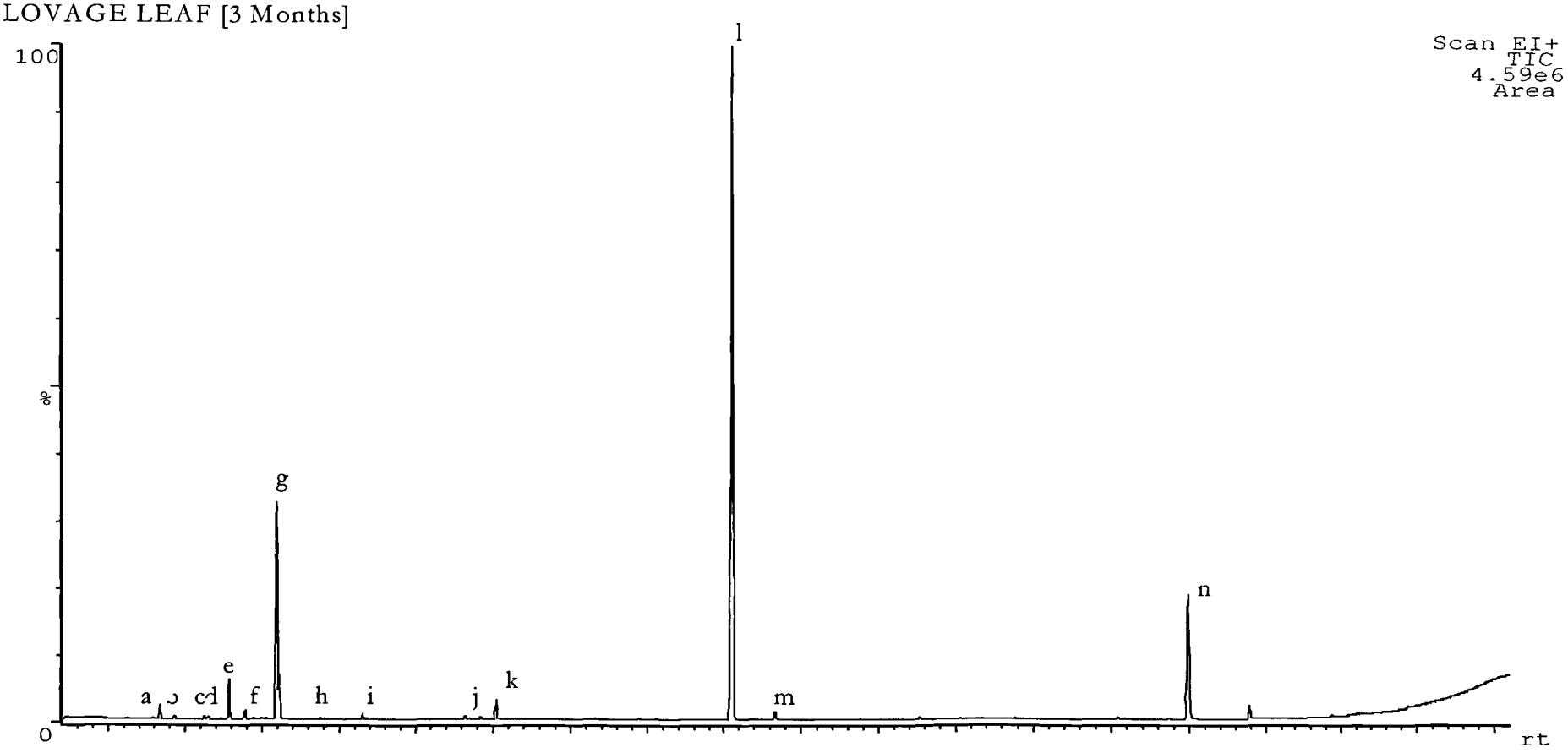
Peak Key: a: Eugenol; **b**: β-Caryophyllene; **c**: α-Humulene; **d**: Eugenyl acetate; **e**: β-Citronellol.

Appendix 5. GC-MS chromatogram of geranium volatile oil.



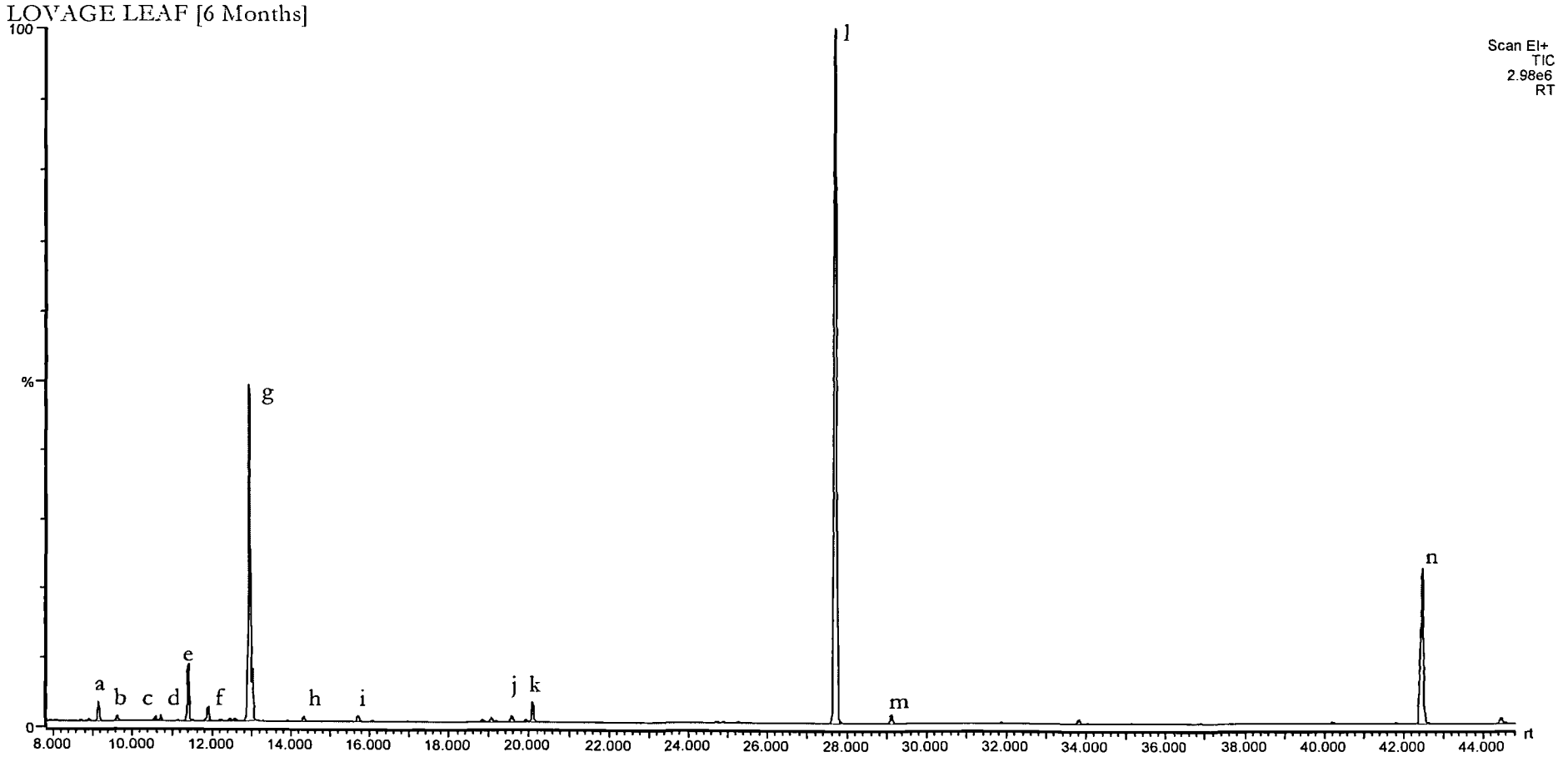
Peak Key: a: Linalool; **b**: Menthone; **c**: *iso*-Menthone; **d**: α-Terpineol; **e**: Citronellol; **f**: *trans*-Geraniol; **g**: Citronellyl formate; **h**: unidentified; **i**: unidentified; **j**: Aromadendrene; **k**: unidentified; **l**: unidentified; **m**: unidentified; **n**: Geranyl tiglate.

Appendix 6. GC-MS chromatogram of lovage leaf (3 months) volatile oil.



Peak Key: a: α-Pinene; **b**: Camphene; **c**: Sabinene; **d**: β-Pinene; **e**: Myrcene; **f**: α-Phellandrene; **g**: Unknown; **h**: γ-Terpinene; **i**: Terpinolene; **j**: Terpinen-4-ol; **k**: α-Terpineol; **l**:*p*-Menth-1-en-8-ol acetate; **m**: Neryl acetate **n**: Unknown.

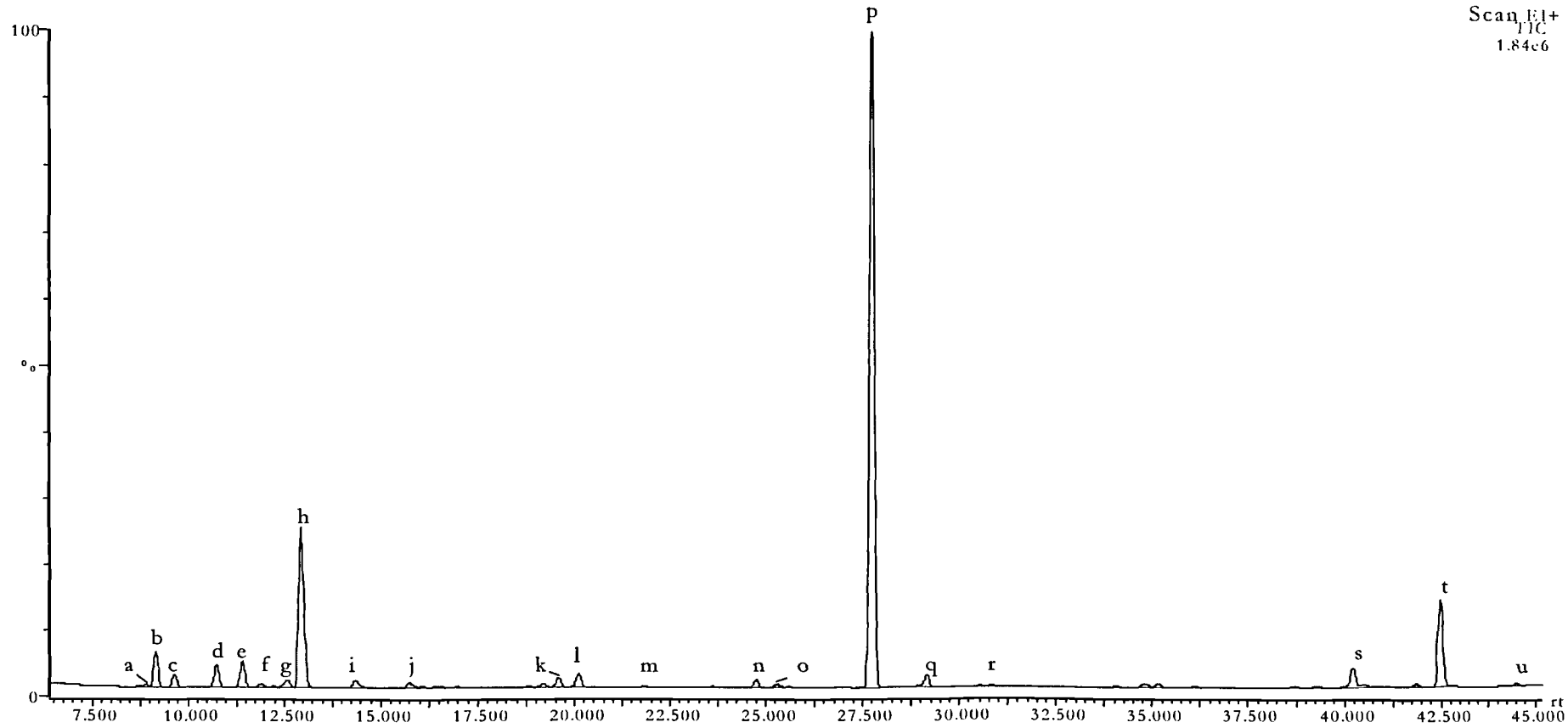
Appendix 7. GC-MS chromatogram of lovage leaf (6 months) volatile oil.



Peak Key: **a**: α-Pinene; **b**: Camphene; **c**: Sabinene; **d**: -Pinene; **e**: Myrcene; **f**: α-Phellandrene; **g**: Unknown; **h**: γ-Terpinene; **i**: Terpinolene; **j**: Terpinen-4-ol; **k**: α-Terpineol; **l**: *p*-Menth-1-en-8-ol acetate; **m**: Neryl acetate **n**: Unknown.

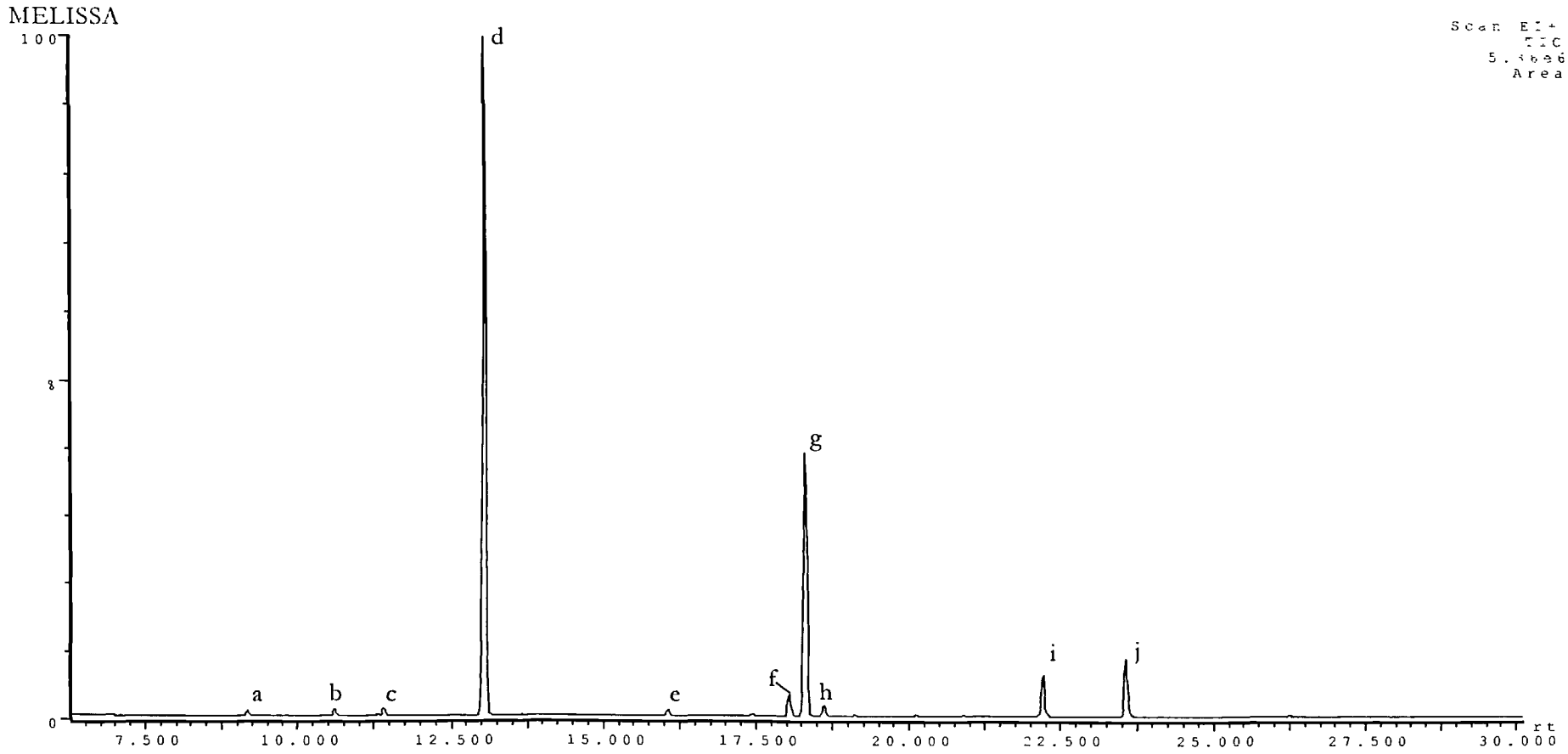
Appendix 8. GC-MS chromatogram of lovage stem volatile oil.

LOVAGE STEM



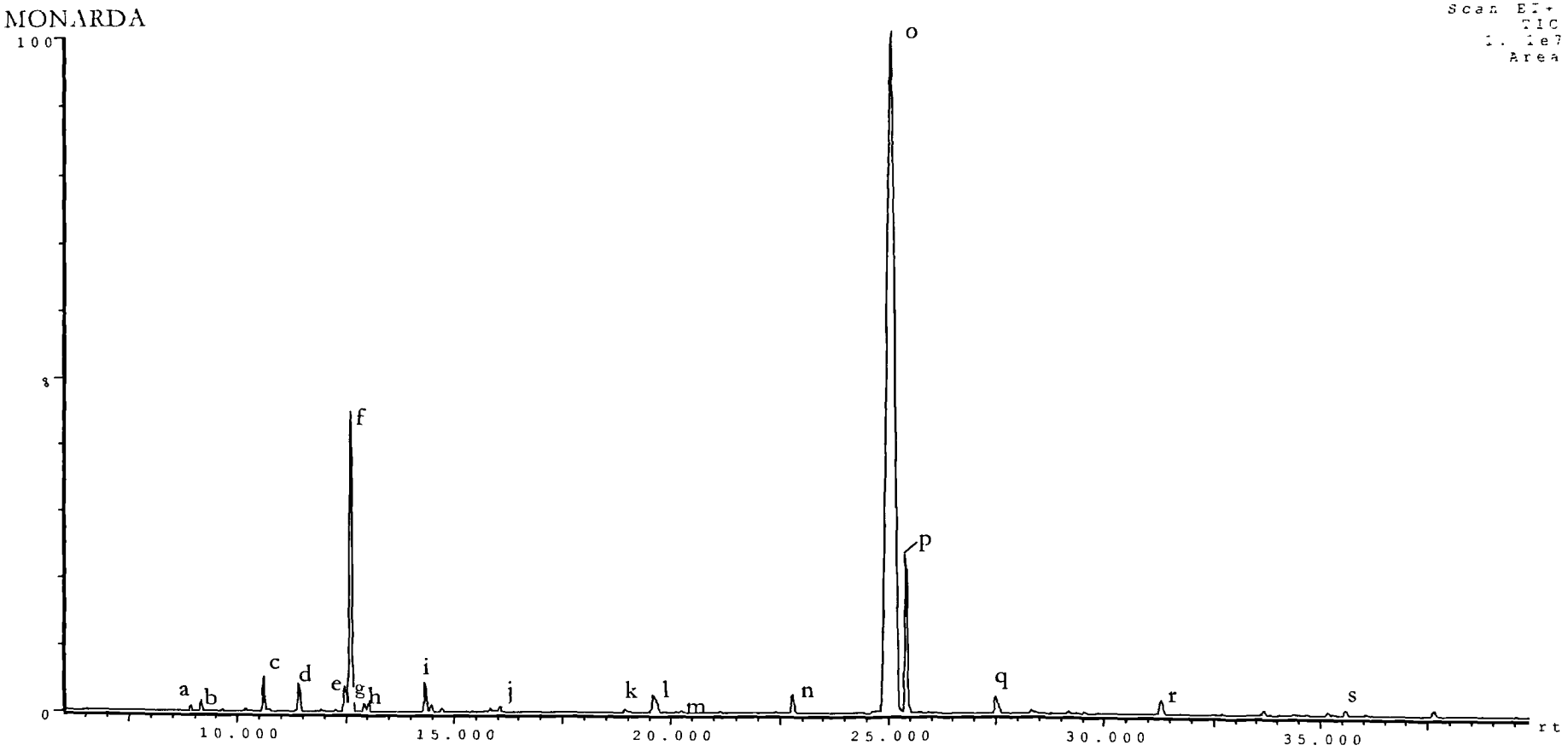
Peak Key: a: α-Thujene; **b**: α-Pinene; **c**: Camphene; **d**: β-Pinene; **e**: Mycrene; **f**: α-Phellandrene; **g**: *p*-Cymene; **h**: Unknown; **i**: γ-Terpinene; **j**: Terpinolene; **k**: Terpinen-4-ol; **l**: α-Terpineol; **m**: Unknown; **n**: Bornyl Acetate; **o**: Unknown; **p**: α-Terpinyl Acetate; **q**: Nerol Acetate; **r**: Unknown; **s**: Unknown; **t**: Unknown; **u**: Unknown.

Appendix 9. GC-MS chromatogram of melissa volatile oil.



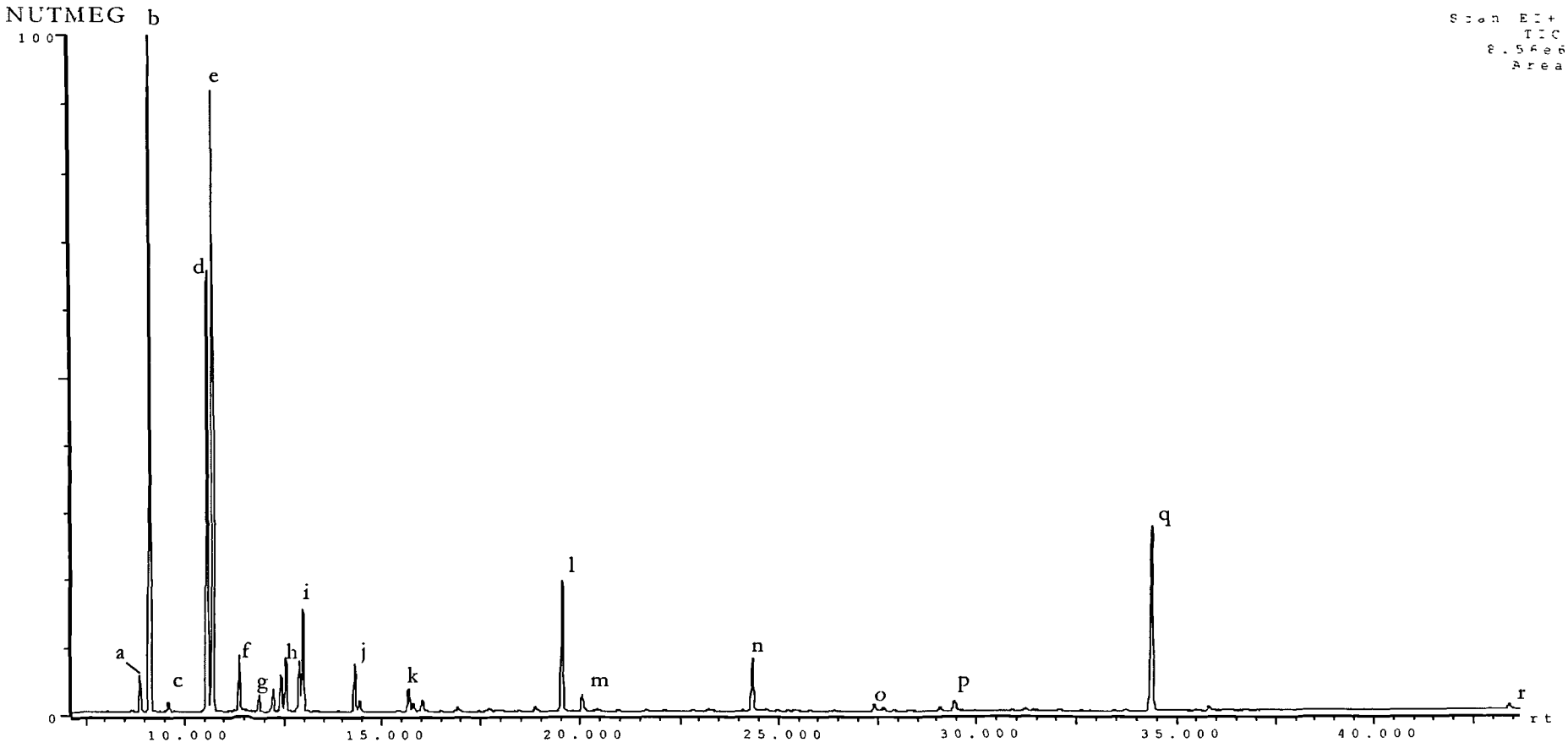
Peak Key: a: α-Pinene; **b**: Sabinene; **c**: Myrcene; **d**: Limonene; **e**: Linalool; **f**: Isopulegol; **g**: Citronellal; **h**: Unknown; **i**: Nerol; **j**: Geraniol.

Appendix 10. GC-MS chromatogram of monarda volatile oil.



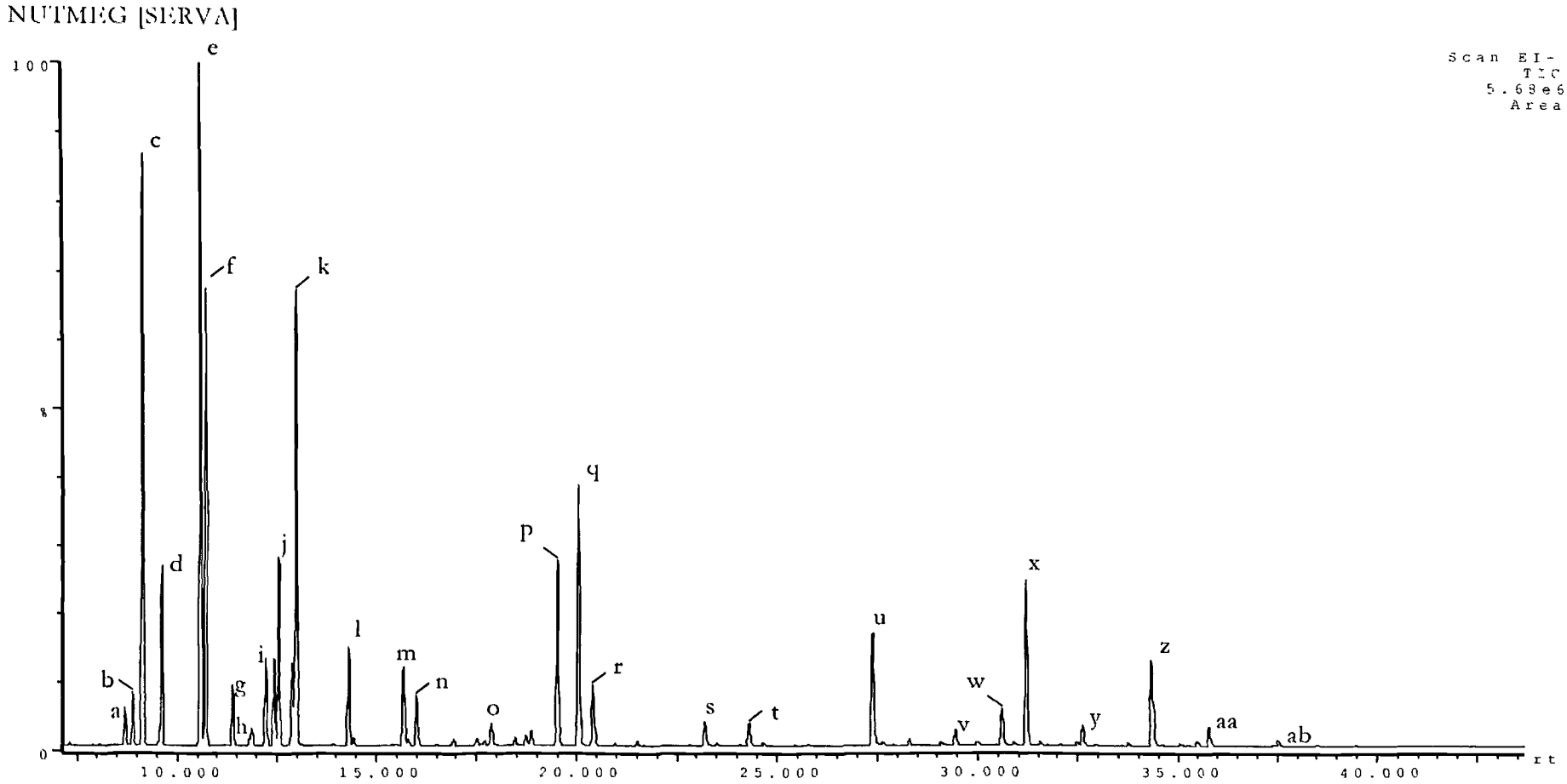
Peak Key: a: α-Thujene; **b**: α-Pinene; **c**: 7-Octen-4-ol; **d**: Myrcene; **e**: α-Terpinene; **f**: *p*-Cymene; **g**: 1,8-Cineole; **h**: Limonene; **i**:γ-Terpinene; **j**: Linalool; **k**: Borneol; **l**: Terpinen-4-ol; **m**: α-Terpineol; **n**: Carvacrol methyl ether; **o**: Thymol; **p**: Carvacrol; **q**: Unknown; **r**: β-Caryophyllene; **s**: δ-Cadinene.

Appendix 11. GC-MS chromatogram of nutmeg volatile oil.



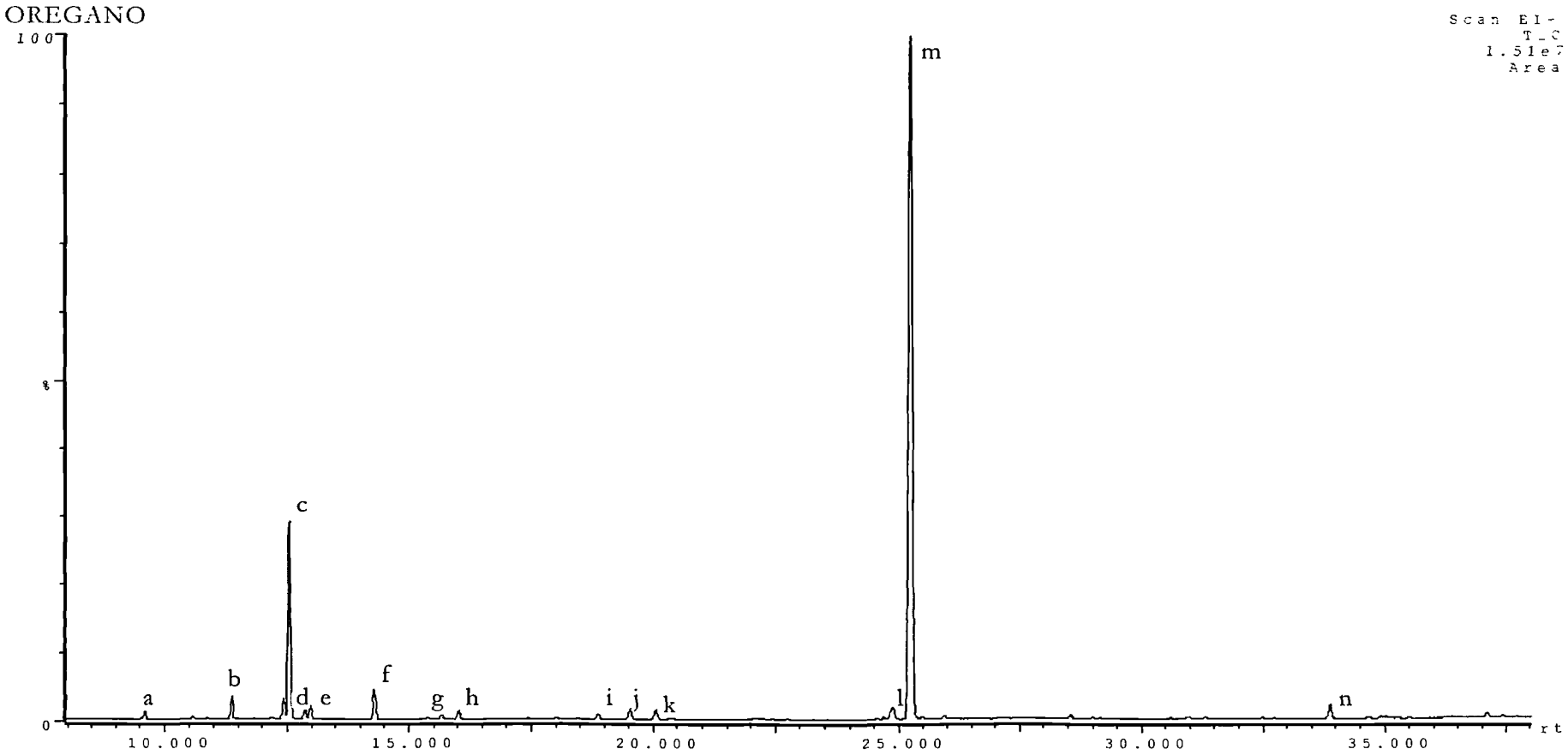
Peak Key: a: α-Thujene; **b**: α-Pinene; **c**: Camphene; **d**: (+)-Sabinene; **e**: β-Pinene; **f**: Myrcene; **g**: R(-)-α-Phellandrene; **h**: *p*-Cymene; **i**: R(-)-Limonene; **j**: γ-Terpineol; **k**: Terpinolene; **l**: 3-Cyclohexan-1-ol, **m**: α-Terpineol; **n**: 1,3-Benzodioxole; **o**: Eugenol; **p**: Methyl eugenol; **q**: Myristicin; **r**: Myristic acid methyl ester.

Appendix 12. GC-MS chromatogram of nutmeg [Serva] volatile oil.



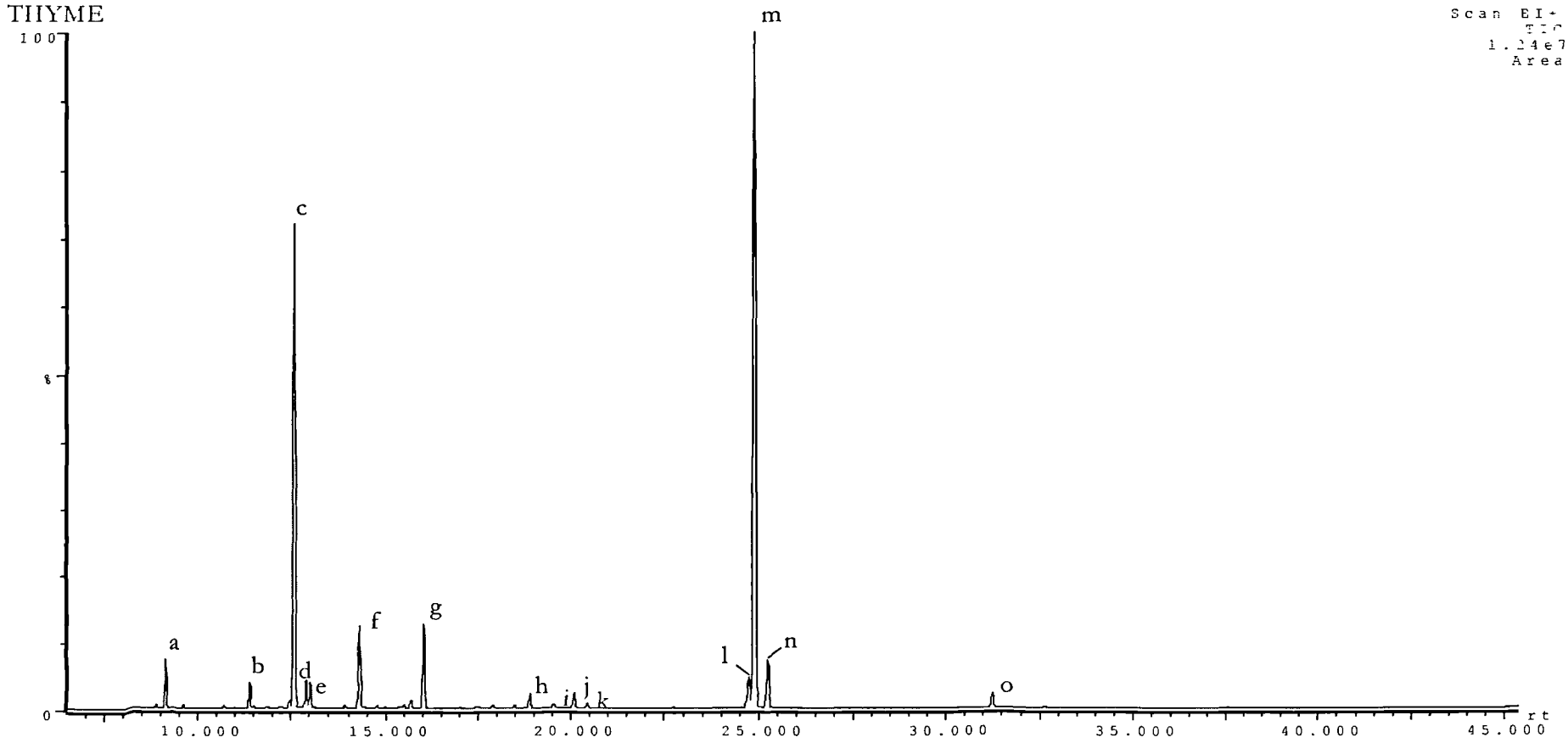
Peak Key: **a**: Tricyclene; **b**: α-Thujene; **c**: α-Pinene; **d**: Camphene; **e**: (+)-Sabinene; **f**: β-Pinene; **g**: Myrcene; **h**: R(-)-α-Phellandrene; **i**: Terpinen-4-ol; **j**: p-Cymene; **k**: R(-)-Limonene; **l**: γ-Terpinene; **m**: Terpinolene; **n**: Linalool; **o**: Cyclohexanol; **p**: Terpinen-4-ol; **q**: α-Terpineol; **r**: γ-Terpineol; **s**: *trans*-Geraniol; **t**: unidentified; **u**: Eugenol; **v**: unidentified; **w**: unidentified; **x**: β-Caryophyllene; **y**: α-Humulene; **z**: Myristicin; **aa**: unidentified; **ab**: Caryophyllene oxide.

Appendix 13. GC-MS chromatogram of oregano volatile oil.



Peak Key: **a**: Camphene; **b**: Myrcene; **c**: p-Cymene; **d**: 1,8-Cineole; **e**: R(-)-Limonene ; **f**: γ-Terpinene; **g**: Terpinolene; **h**: Linalool ; **i**: Borneol; **j**: Terpinen-4-ol; **k**: α-Terpineol; **l**: Thymol; **m**: Carvacrol; **n**: unidentified.

Appendix 14. GC-MS chromatogram of thyme volatile oil.



Peak Key: a: α-Pinene; **b**: Myrcene; **c**: *p*-Cymene; **d**: 1,8-Cineole; **e**: R(-)-Limonene; **f**: γ-Terpinene; **g**: Linalool; **h**: Borneol; **i**: Terpinen-4-ol; **j**: α-Terpineol; **k**: γ-Terpineol; **l**: Bornyl acetate; **m**: Thymol; **n**: Carvacrol; **o**: Caryophyllene oxide.

Appendix 15. Data for the antimycotic activity of volatile oils used in this study against *Aspergillus flavus*, *Aspergillus niger* and *Fusarium culmorum*.

Activity of volatile oils from black pepper, clove, melissa, oregano and thyme against* Aspergillus flavus[a] *(inhibition index as a percentage relative to control flasks).

	Concentration of oils (μL mL^{-1} YES broth)						
	1	2	5	10	20	50	100
Black Pepper	77	81	83	84	85	88	92
Clove	57	71	82	87	88	93	97
Melissa	48	76	78	83	83	86	90
Oregano	34	37	65	76	83	88	95
Thyme	59	70	71	82	83	86	91

[a] The source of the fungal strain was IMI 89717.

Activity of volatile oils from black pepper, clove, melissa, oregano and thyme against* Aspergillus niger[b] *(inhibition index as a percentage relative to control flasks).

	Concentration of oils (μL mL^{-1} YES broth)						
	1	2	5	10	20	50	100
Black Pepper	68	70	73	85	95	97	98
Clove	60	72	76	80	80	82	92
Melissa	79	81	84	86	89	92	92
Oregano	76	77	80	87	82	92	97
Thyme	68	77	80	87	73	81	95

[b] The source of the fungal strain was IMI 17454.

Activity of volatile oils from black pepper, clove, melissa, oregano and thyme against* Fusarium culmorum[c] *(inhibition index as a percentage relative to control flasks).

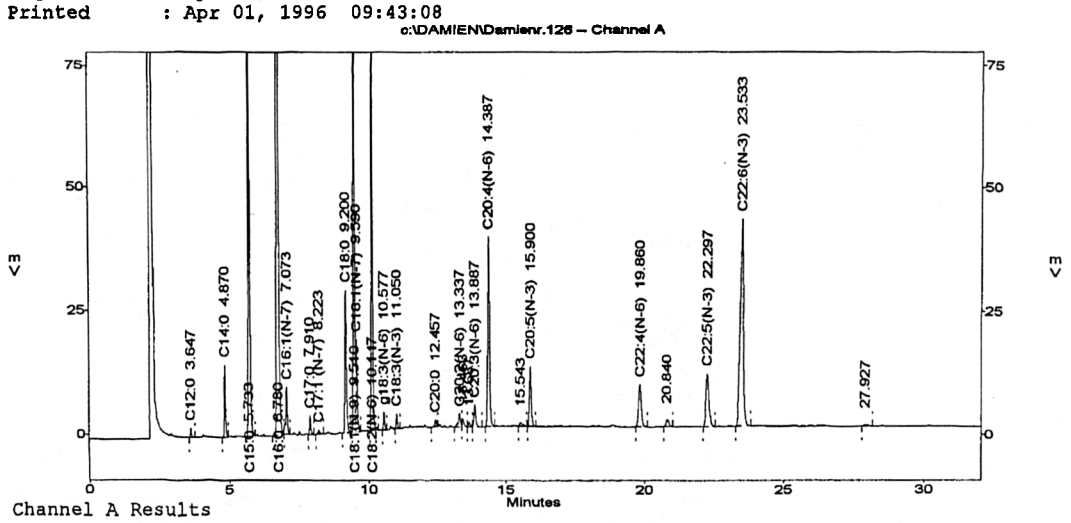
	Concentration of oils (μL mL^{-1} YES broth)						
	1	2	5	10	20	50	100
Black Pepper	45	48	50	76	83	86	90
Clove	52	52	55	57	87	93	96
Melissa	55	60	63	64	86	90	91
Oregano	52	53	54	70	85	91	95
Thyme	63	64	66	70	85	89	93

[c] The source of the fungal strain was IMI 307847.

Appendix 16. Gas chromatographic trace and percentage composition of the major fatty acids present in the triacylglyceride lipid fraction from the liver of a neonatal rat feed thyme volatile oil at 3.5mg Kg^{-1} day^{-1} *in utero*, as described in section 6.3. Conditions of separation were as described in Section 6.2.2. C15:0 = Internal standard.

GC4-RT'S UPDATED 06/02/96 Column No. 2523-8

File : c:\DAMIEN\Damienr.126
Method : c:\ezchrom\methods\4-260396.met
Sample ID : DAMIENR.126
Acquired : Apr 01, 1996 09:10:47
Printed : Apr 01, 1996 09:43:08



Channel A Results

Peak No.	Peak Name	Time	RRT(16:0)	AREA	Norm %
1	C12:0	3.647	0.316	5097	0.16
2	C14:0	4.870	0.583	40013	1.23
3	C15:0	5.733	0.771	516691	0.00
4	C16:0	6.780	1.000	1559234	47.96
5	C16:1(N-7)	7.073	1.064	34606	1.06
6	C17:0	7.910	1.247	12016	0.37
7	C17:1 (N-7)	8.223	1.315	3653	0.11
8	C18:0	9.200	1.528	105935	3.26
9	C18:1(N-9)	9.510	1.596	348999	10.73
10	C18:1(N-7)	9.590	1.614	61815	1.90
11	C18:2(N-6)	10.147	1.735	315273	9.70
12	g18:3(N-6)	10.577	1.829	11705	0.36
13	C18:3(N-3)	11.050	1.932	9988	0.31
14	C20:0	12.457	2.239	5244	0.16
15	C20:2(N-6)	13.337	2.432	13746	0.42
16		13.463	0.000	7182	0.00
17		13.687	0.000	4279	0.00
18	C20:3(N-6)	13.887	2.552	20117	0.62
19	C20:4(N-6)	14.387	2.661	175541	5.40
20		15.543	0.000	5590	0.00
21	C20:5(N-3)	15.900	2.991	58028	1.78
22	C22:4(N-6)	19.860	3.856	58396	1.80
23		20.840	0.000	10110	0.00
24	C22:5(N-3)	22.297	4.388	81147	2.50
25	C22:6(N-3)	23.533	4.658	330580	10.17
26		27.927	0.000	3184	0.00
Totals :				3798169	100.00

Channel A Results

FAME/IS Ratio
6.3448

Appendix 17. Fatty acid composition (percentage of major fatty acids by weight) of the triacylglyceride fraction extracted from adipose of pregnant rats fed different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	0.09±0.0	0.06±0.01	0.08±0.00	0.07±0.00
14:0	1.10±0.02	1.21±0.12	1.18±0.07	1.11±0.02
16:0	27.1±2.9	26.6±1.79	24.3±0.59	25.2±1.54
17:0	0.29±0.02	0.26±0.02	0.26±0.01	0.29±0.01
18:0	3.87±0.27	3.67±0.26	3.46±0.06	3.92±0.18
20:0	tr.	tr.	tr.	tr.
Total Saturates	***32.6***	***31.8***	***29.3***	***30.6***
14:1 *n-5*	tr.	tr.	tr.	tr.
16:1 *n-7*	3.87±0.41	4.36±0.26	3.91±0.22	3.71±0.20
17:1 *n-7*	0.23±0.01	0.25±0.01	0.25±0.01	0.24±0.02
18:1 *n-9*	25.1±0.12	28.3±0.50[b]	27.0±0.20[b]	23.6±3.94
18:1 *n-7*	3.02±0.01	3.67±0.14[b]	3.11±0.11	3.04±0.06
20:1 *n-9*	0.36±0.06	0.19±0.02[a]	0.28±0.07	0.33±0.07
22:1 *n-9*	0.07±0.01	0.06±0.01	tr.	0.06±0.00
Total Monoenes	***32.6***	***36.8***	***34.6***	***30.9***
18:2 *n-6*	32.4±0.05	30.3±1.74	31.2±0.56	34.2±2.74
18:3 *n-6*	0.13±0.01	0.09±0.00[b]	0.16±0.02	0.12±0.02
20:2 *n-6*	0.29±0.01	0.19±0.01[b]	0.22±0.04	0.20±0.05
20:3 *n-6*	0.21±0.00	0.12±0.00[c]	0.17±0.01	0.18±0.02
20:4 *n-6*	0.73±0.04	0.47±0.06[b]	0.58±0.04[a]	0.64±0.07
22:4 *n-6*	0.19±0.01	0.14±0.02[b]	0.15±0.01[b]	0.17±0.02
Total n-6 PUFAs	***33.9***	***31.4***	***32.5***	***35.5***
18:3 *n-3*	2.30±0.10	1.70±0.10[b]	1.96±0.07	2.14±0.13
18:4 *n-3*	tr.	tr.	tr.	tr.
20:5 *n-3*	0.30±0.01	0.27±0.02	0.25±0.01[a]	0.30±0.00
22:3 *n-3*	tr.	tr.	tr.	tr.
22:5 *n-3*	0.68±0.06	0.50±0.07[a]	0.52±0.02[a]	0.55±0.06[a]
22:6 *n-3*	1.53±0.07	1.04±0.08[b]	1.11±0.04[c]	1.24±0.14[a]
Total n-3 PUFAs	***4.85***	***3.59***	***3.88***	***4.27***
Total PUFA	***38.8***	***34.9***	***36.4***	***39.8***
U/S ratio[1]	***1.00***	***1.16***	***1.18***	***1.01***
P/S ratio[2]	***1.19***	***1.10***	***1.24***	***1.30***
n-6/n-3 ratio[3]	***6.98***	***8.75***	***8.38***	***8.31***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids; [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 18. Fatty acid composition (percentage of major fatty acids by weight) of the phospholipid fraction extracted from brain of pregnant rats fed different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	0.11±0.01	0.10±0.01	0.11±0.01	0.13±0.01
16:0	19.43±0.48	19.83±0.42	20.65±0.51	21.15±0.85
17:0	0.23±0.01	0.21±0.01	0.22±0.01	0.45±0.28
18:0	20.4±0.56	19.9±0.14	20.2±0.45	21.2±0.0
20:0	0.54±0.03	0.50±0.03	0.51±0.04	0.42±0.03
Total Saturates	***40.7***	***40.6***	***41.7***	***43.4***
14:1 *n-5*	tr.	tr.	tr.	tr.
16:1 *n-7*	0.51±0.04	0.44±0.03	0.45±0.06	0.38±0.01
17:1 *n-7*	1.62±0.26	1.79±0.31	0.42±0.10[c]	1.37±0.12
18:1 *n-9*	22.1±0.27	21.5±0.42	22.0±0.24	21.7±0.17
18:1 *n-7*	4.52±0.06	4.55±0.13	4.58±0.07	4.35±0.21
20:1 *n-9*	3.22±0.07	3.04±0.21	2.89±0.07[b]	2.02±0.30[c]
22:1 *n-9*	0.22±0.01	0.24±0.01	0.20±0.01	tr.[c]
Total Monoenes	***32.2***	***31.6***	***30.6***	***29.8***
18:2 *n-6*	0.82±0.05	0.77±0.04	0.84±0.04	0.70±0.05
18:3 *n-6*	tr.	tr.	tr.	tr.
20:2 *n-6*	0.17±0.01	0.19±0.02	0.18±0.01	tr.[c]
20:3 *n-6*	0.40±0.02	0.41±0.01	0.39±0.01	0.35±0.02
20:4 *n-6*	8.67±0.38	9.02±0.15	8.37±0.40	8.59±0.42
22:4 *n-6*	2.90±0.15	2.98±0.08	2.80±0.23	2.37±0.06
Total n-6 PUFAs	***12.9***	***13.4***	***12.6***	***12.0***
18:3 *n-3*	0.14±0.03	0.12±0.01	0.21±0.03[b]	0.19±0.05[a]
18:4 *n-3*	tr.	tr.	tr.	tr.
20:5 *n-3*	0.41±0.03	0.37±0.03	0.36±±0.02	0.37±0.04
22:3 *n-3*	tr.	tr.	tr.	tr.
22:5 *n-3*	0.20±0.02	0.22±0.01	0.21±0.01	0.66±0.10[c]
22:6 *n-3*	13.8±0.58	14.2±0.30	13.6±0.58	13.8±0.37
Total n-3 PUFAs	***14.5***	***14.9***	***14.4***	***14.9***
Total PUFA	***27.5***	***28.3***	***27.0***	***27.0***
U/S ratio[1]	***0.79***	***0.78***	***0.73***	***0.69***
P/S ratio[2]	***0.68***	***0.70***	***0.65***	***0.62***
n-6/n-3 ratio[3]	***0.89***	***0.90***	***0.88***	***0.81***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] P≤0.05; [b] P≤0.01; [c] P≤0.001. tr.:≤0.05% weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 19. Fatty acid composition (percentage of major fatty acids by weight) of the free fatty acid fraction extracted from heart of pregnant rats fed different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	0.31±0.00	0.39±0.09	0.41±0.10	0.78±0.26
16:0	19.8±3.37	23.4±2.09	18.9±0.64	22.9±2.97
17:0	0.51±0.09	0.48±0.04	0.48±0.06	0.50±0.00
18:0	20.4±1.25	18.8±1.10	20.4±1.64	19.5±0.37
20:0	0.14±0.03	0.17±0.02	0.15±0.00	tr.[c]
Total Saturates	***41.1***	***43.2***	***40.4***	***43.4***
14:1 *n-5*	tr.	tr.	tr.	tr.
16:1 *n-7*	0.76±0.09	0.61±0.06	0.75±0.09±4	1.11±0.21
17:1 *n-7*	0.21±0.04.	0.32±0.05	0.24±0.03	0.41±0.00
18:1 *n-9*	8.29±0.73	10.9±0.88	11.1±1.09	9.95±0.08
18:1 *n-7*	3.50±0.07	3.76±0.17	3.87±0.21	3.38±0.02
20:1 *n-9*	0.31±0.01	0.37±0.02	0.46±0.07	0.33±0.00
22:1 *n-9*	tr.	tr.	tr.	tr.
Total Monoenes	***13.1***	***16.0***	***16.4***	***15.2***
18:2 *n-6*	19.7±3.46	17.4±1.49	17.6±0.77	16.4±0.10
18:3 *n-6*	0.14±0.04	0.22±0.01[b]	0.20±0.03[a]	0.20±0.04[a]
20:2 *n-6*	1.58±0.24	2.56±0.46[a]	0.96±0.15	2.14±0.01[a]
20:3 *n-6*	0.46±0.06	0.40±0.02	0.37±0.02	0.45±0.00
20:4 *n-6*	11.4±1.45	10.9±0.80	10.7±0.40	10.5±0.90
22:4 *n-6*	0.52±0.14	0.39±0.03	0.45±0.02	0.54±0.00
Total n-6 PUFAs	***33.8***	***31.8***	***30.2***	***30.3***
18:3 *n-3*	0.40±0.08	0.35±0.04	0.36±0.03	0.47±0.00
18:4 *n-3*	tr.	tr.	tr.	tr.
20:5 *n-3*	0.24±0.09	0.33±0.01	0.27±0.02	0.36±0.00
22:3 *n-3*	tr.	tr.	tr.	tr.
22:5 *n-3*	2.05±0.32	2.25±0.22	2.38±0.28	2.21±0.30
22:6 *n-3*	9.12±0.86	10.6±1.02	10.16±0.90	9.48±1.01
Total n-3 PUFAs	***11.8***	***13.6***	***13.2***	***12.5***
Total PUFA	***45.7***	***45.4***	***43.4***	***42.8***
U/S ratio[1]	***0.32***	***0.37***	***0.41***	***0.35***
P/S ratio[2]	***1.11***	***1.05***	***1.08***	***0.97***
n-6/n-3 ratio[3]	***2.86***	***2.34***	***2.29***	***2.42***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05% weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 20. Fatty acid composition (percentage of major fatty acids by weight) of the phospholipid fraction extracted from heart of pregnant rats fed different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	0.12±0.03	0.10±0.02	tr.	0.12±0.00
16:0	13.2±0.84	13.1±0.99	13.9±0.73	13.1±0.13
17:0	0.38±0.03	0.38±0.02	0.37±0.01	0.36±0.02
18:0	26.6±1.22	23.8±0.67[a]	24.2±0.72	25.9±1.77
20:0	0.15±0.00	0.16±0.01	0.14±0.00	0.18±0.01[a]
Total Saturates	***40.4***	***37.6***	***38.7***	***39.7***
14:1 *n-5*	tr.	tr.	tr.	tr.
16:1 *n-7*	0.35±0.07	0.36±0.02	0.44±0.04	0.39±0.04
17:1 *n-7*	0.33±0.04	0.39±0.02	0.46±0.05	0.50±0.07
18:1 *n-9*	3.70±0.57	3.06±0.12	3.09±0.14	3.02±0.19
18:1 *n-7*	3.90±0.22	3.56±0.18	3.59±0.13	3.78±0.28
20:1 *n-9*	0.28±0.05	0.18±0.02[a]	0.22±0.05	0.29±0.02
22:1 *n-9*	0.09±0.01	0.09±0.02	tr.	0.14±0.00
Total Monoenes	***8.65***	***7.64***	***7.80***	***8.12***
18:2 *n-6*	16.9±0.88	16.7±0.93	16.8±0.68	13.9±0.63[a]
18:3 *n-6*	0.20±0.05	0.15±0.02	0.15±0.00	0.13±0.00
20:2 *n-6*	0.27±0.03	0.24±0.01	0.23±0.02	0.34±0.04
20:3 *n-6*	0.36±0.01	0.38±0.01	0.35±0.01	0.35±0.01
20:4 *n-6*	16.4±0.89	17.2±0.53	16.7±0.05	16.9±0.84
22:4 *n-6*	0.40±0.04	0.39±0.02	0.38±0.02	0.51±0.05
Total n-6 PUFAs	***34.6***	***35.1***	***34.6***	***32.2***
18:3 *n-3*	0.08±0.00	0.10±0.01	0.09±0.00	0.18±0.06
18:4 *n-3*	tr.	tr.	tr.	tr.
20:5 *n-3*	0.22±0.01	0.25±0.03	0.24±0.00	0.21±0.01
22:3 *n-3*	tr.	tr.	tr.	tr.
22:5 *n-3*	2.17±0.21	2.68±0.17[a]	2.66±0.11[a]	2.52±0.20
22:6 *n-3*	14.1±1.93	16.9±1.21	16.5±0.78	17.4±1.96
Total n-3 PUFAs	***16.6***	***19.9***	***19.5***	***20.3***
Total PUFA	***51.1***	***55.0***	***54.1***	***52.4***
U/S ratio[1]	***0.21***	***0.20***	***0.20***	***0.21***
P/S ratio[2]	***1.27***	***1.46***	***1.40***	***1.32***
n-6/n-3 ratio[3]	***2.08***	***1.76***	***1.77***	***1.59***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05% weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 21. Fatty acid composition (percentage of major fatty acids by weight) of the triacylglyceride fraction extracted from kidney of pregnant rats fed different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	0.09±0.02
14:0	1.20±0.10	1.17±0.07	1.28±0.09	1.11±0.04
16:0	26.9±1.73	26.0±1.28	27.3±1.37	24.9±0.70
17:0	0.26±0.01	0.32±0.03	0.23±0.01	0.31±0.02
18:0	4.32±0.08	5.24±1.35	4.36±0.15	5.14±0.14[b]
20:0	0.09±0.01	0.12±0.01	0.09±0.01	0.12±0.01[a]
Total Saturates	***32.8***	***32.9***	***33.2***	***31.7***
14:1 *n-5*	0.06±0.01	0.07±0.00	0.07±0.02	0.07±0.00
16:1 *n-7*	4.92±0.13	4.08±0.43	5.89±0.10[c]	4.04±0.53
17:1 *n-7*	0.25±0.01	0.36±0.10	0.27±0.08	0.24±0.01
18:1 *n-9*	28.3±1.04	28.9±1.35	29.7±0.37	28.4±0.42
18:1 *n-7*	2.79±0.13	2.85±0.08	2.86±0.02	2.79±0.06
20:1 *n-9*	0.30±0.05	0.58±0.06[b]	0.45±0.15	0.40±0.03
22:1 *n-9*	tr.	tr.	tr.	tr.
Total Monoenes	***36.6***	***36.9***	***39.2***	***35.9***
18:2 *n-6*	27.2±0.23	25.2±1.17	24.6±1.07[a]	26.3±0.04
18:3 *n-6*	0.11±0.02	0.11±0.01	0.07±0.02	0.18±0.00[b]
20:2 *n-6*	tr.	0.39±0.06[c]	tr.	0.40±0.08[c]
20:3 *n-6*	0.18±0.01	0.17±0.02	0.14±0.01[a]	0.20±0.00
20:4 *n-6*	1.09±0.10	1.01±0.12	0.85±0.06	1.61±0.10[b]
22:4 *n-6*	0.13±0.01	0.12±0.01	0.10±0.01[a]	0.14±0.02
Total n-6 PUFAs	***28.7***	***27.0***	***25.8***	***28.9***
18:3 *n-3*	1.60±0.07	1.24±0.04[b]	1.48±0.12	1.56±0.05
18:4 *n-3*	tr.	tr.	tr.	tr.
20:5 *n-3*	0.14±0.06	0.25±0.03[a]	0.07±0.05	0.32±0.05[b]
22:3 *n-3*	tr.	tr.	tr.	tr.
22:5 *n-3*	0.26±0.13	0.44±0.03	0.19±0.09	0.38±0.01
22:6 *n-3*	0.74±0.25	0.85±0.08	0.70±0.01	0.91±0.05
Total n-3 PUFAs	***2.74***	***2.78***	***2.44***	***3.17***
Total PUFA	***31.5***	***29.8***	***28.2***	***32.0***
U/S ratio[1]	***1.12***	***1.12***	***1.18***	***1.13***
P/S ratio[2]	***0.96***	***0.91***	***0.85***	***1.01***
n-6/n-3 ratio[3]	***10.5***	***9.71***	***10.6***	***9.12***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05% weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 22. Fatty acid composition (percentage of major fatty acids by weight) of the free fatty acid fraction extracted from kidney of pregnant rats fed different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	0.47±0.02	0.40±0.03	0.40±0.09	0.46±0.01
16:0	17.7±0.56	18.8±0.80	19.1±0.62	18.5±0.35
17:0	0.34±0.05	0.41±0.04	0.33±0.05	0.43±0.06
18:0	13.0±1.03	13.7±1.08	13.1±0.29	13.3±0.05
20:0	0.17±0.02	0.18±0.11	0.17±0.01	0.20±0.06
Total Saturates	***31.7***	***33.5***	***33.1***	***32.9***
14:1 *n-5*	tr.	tr.	tr.	tr.
16:1 *n-7*	1.36±0.16	1.25±0.13	1.42±0.08	1.39±0.20
17:1 *n-7*	0.15±0.01	0.33±0.06[c]	0.23±0.05[b]	0.22±0.06
18:1 *n-9*	16.4±1.34	14.1±0.84	14.9±0.38	14.0±0.86
18:1 *n-7*	2.46±0.10	2.35±0.06	2.46±0.05	2.10±0.06[a]
20:1 *n-9*	0.49±0.03	0.39±0.02	0.53±0.02	0.37±0.00[a]
22:1 *n-9*	tr.	tr.	tr.	tr.
Total Monoenes	***20.9***	***18.4***	***19.6***	***18.0***
18:2 *n-6*	18.4±1.70	16.0±0.50	15.8±0.28	15.8±0.48
18:3 *n-6*	0.21±0.09	0.14±0.00	0.25±0.02	0.33±n.d.
20:2 *n-6*	0.58±0.08	0.60±0.05	0.53±0.03	0.65±0.09
20:3 *n-6*	0.88±0.07	0.94±0.03	1.00±0.05	1.01±0.06
20:4 *n-6*	19.2±2.58	20.2±1.15	20.9±0.91	21.0±0.75
22:4 *n-6*	0.38±0.03	0.41±0.02	0.40±0.04	0.49±0.08
Total n-6 PUFAs	***39.6***	***38.3***	***38.9***	***39.3***
18:3 *n-3*	0.79±0.15	0.47±0.06[a]	0.55±0.04[a]	0.75±0.19
18:4 *n-3*	tr.	tr.	tr.	tr.
20:5 *n-3*	1.64±0.03	1.49±0.09	1.60±0.04	1.63±0.19
22:3 *n-3*	tr.	tr.	tr.	tr.
22:5 *n-3*	0.95±0.06	1.32±0.09[b]	1.21±0.04[b]	1.05±0.07
22:6 *n-3*	6.20±0.60	6.69±0.47	6.79±0.21	7.20±0.39
Total n-3 PUFAs	***9.58***	***9.97***	***10.15***	***10.63***
Total PUFA	***49.2***	***48.3***	***49.0***	***49.9***
U/S ratio[1]	***0.66***	***0.55***	***0.59***	***0.55***
P/S ratio[2]	***1.55***	***1.44***	***1.48***	***1.52***
n-6/n-3 ratio[3]	***4.13***	***3.84***	***3.83***	***3.70***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05% weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 23. Fatty acid composition (percentage of major fatty acids by weight) of the phospholipid fraction extracted from kidney of pregnant rats fed different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	0.26±0.02	0.23±0.02	0.22±0.02	0.22±0.02
16:0	27.6±1.23	25.0±1.19	26.9±1.21	25.2±0.54
17:0	0.43±0.01	0.45±0.04	0.38±0.02[a]	0.41±0.03
18:0	18.8±0.89	19.6±0.55	18.3±0.35	19.6±0.74
20:0	0.29±0.03	0.37±0.06	0.28±0.02	0.30±0.03
Total Saturates	***47.4***	***45.7***	***46.0***	***45.8***
14:1 *n-5*	tr.	tr.	tr.	tr.
16:1 *n-7*	0.85±0.10	0.88±0.09	0.86±0.03	0.76±0.05
17:1 *n-7*	0.44±0.06	0.42±0.07	0.47±0.05	0.50±0.08
18:1 *n-9*	9.83±0.49	9.53±0.23	8.97±0.15	8.84±0.39
18:1 *n-7*	3.11±0.16	3.15±0.12	3.44±0.12	2.84±0.19
20:1 *n-9*	0.34±0.09	0.37±0.03	0.29±0.03	0.28±0.04
22:1 *n-9*	tr.	tr.	tr.	tr.
Total Monoenes	***14.6***	***14.4***	***14.0***	***13.2***
18:2 *n-6*	15.1±0.48	15.8±1.04	14.8±0.54	14.6±0.68
18:3 *n-6*	tr.	tr.	tr.	tr.
20:2 *n-6*	0.41±0.04	0.38±0.03	0.37±0.02	0.32±0.03
20:3 *n-6*	0.53±0.03	0.63±0.06	0.63±0.05	0.62±0.04
20:4 *n-6*	16.4±1.35	17.0±0.82	17.1±1.16	18.6±1.19
22:4 *n-6*	0.30±0.02	0.29±0.03	0.32±0.05	0.30±0.02
Total n-6 PUFAs	***32.8***	***34.7***	***33.1***	***34.4***
18:3 *n-3*	0.20±0.03	0.20±0.03	0.22±0.01	0.18±0.02
18:4 *n-3*	tr.	tr.	tr.	tr.
20:5 *n-3*	0.95±0.09	0.92±0.07	0.97±0.03	1.00±0.10
22:3 *n-3*	tr.	tr.	tr.	tr.
22:5 *n-3*	0.54±0.07	1.21±0.67	0.71±0.01[a]	0.54±0.08
22:6 *n-3*	3.63±0.54	4.26±0.34	4.52±0.09	4.43±0.63
Total n-3 PUFAs	***5.32***	***6.59***	***6.42***	***6.15***
Total PUFA	***38.1***	***41.3***	***39.5***	***40.6***
U/S ratio[1]	***0.31***	***0.32***	***0.30***	***0.29***
P/S ratio[2]	***0.80***	***0.90***	***0.86***	***0.89***
n-6/n-3 ratio[3]	***6.17***	***5.27***	***5.12***	***5.60***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05% weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 24. Fatty Acid Composition (Percentage of Major Fatty Acids by Weight) of the Cholesteryl Ester Fraction Extracted from Liver of Pregnant Rats Fed Different Concentrations of Oregano Volatile Oil.

Fatty Acid	*Diet*			
	Control	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	0.40±0.51	0.54±0.08	0.50±0.06	0.69±0.08
16:0	26.5±0.95	28.1±1.35	33.7±2.83	28.2±2.44
17:0	0.50±0.12	0.36±0.06	0.37±0.12	0.51±0.29
18:0	8.34±1.21	10.7±1.15	7.12±1.30	10.3±1.62
20:0	tr.	tr.	tr.	tr.
Total Saturates	***35.8***	***39.8***	***41.7***	***39.7***
14:1 n-5	tr.	tr.	tr.	tr.
16:1 n-7	1.07±0.27	1.44±0.03[b]	1.69±0.22[b]	1.63±0.12[b]
17:1 n-7	0.25±0.10	0.27±0.05	0.13±0.03[b]	0.20±0.03[a]
18:1 n-9	8.89±0.88	12.3±0.90	8.89±1.61	10.2±0.72
18:1 n-7	1.80±0.25	2.11±0.15	1.48±0.11	1.73±0.10
20:1 n-9	0.44±0.86	0.39±0.07	0.29±0.07	0.30±0.07
22:1 n-9	tr.	tr.	tr.	tr.
Total Monoenes	***12.5***	***16.5***	***12.5***	***14.1***
18:2 n-6	23.8±0.81	21.9±1.50	21.6±3.86	23.0±2.86
18:3 n-6	0.41±0.18	0.30±0.07	0.20±0.04	0.23±0.05
20:2 n-6	1.17±0.71	2.09±0.46	3.07±0.78	2.28±0.94
20:3 n-6	0.31±0.02	0.57±0.10	0.25±0.03	0.24±0.03
20:4 n-6	12.2±2.00	10.4±1.03	10.1±0.90	12.6±0.73
22:4 n-6	tr.	tr.	tr.	tr.
Total n-6 PUFAs	***37.9***	***35.3***	***35.2***	***38.3***
18:3 n-3	1.09±0.14	1.36±0.12	1.66±0.40	1.25±0.15
18:4 n-3	tr.	tr.	tr.	tr.
20:5 n-3	1.16±0.06	1.39±0.14	2.17±0.16[a]	1.00±0.08
22:3 n-3	tr.	tr.	tr.	tr.
22:5 n-3	2.70±0.40	1.86±0.18[a]	1.86±0.06[a]	1.67±0.31[a]
22:6 n-3	9.38±1.03	5.50±0.62[b]	6.04±0.56[a]	6.15±0.65[a]
Total n-3 PUFAs	***14.3***	***10.1***	***11.7***	***10.1***
Total PUFA	***52.2***	***45.5***	***46.9***	***48.4***
U/S ratio[1]	***0.35***	***0.42***	***0.30***	***0.36***
P/S ratio[2]	***1.46***	***1.14***	***1.13***	***1.22***
n-6/n-3 ratio[3]	***2.65***	***3.50***	***3.01***	***3.79***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] P<0.05; [b] P<0.01; [c] P<0.001. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 25. Fatty acid composition (percentage of major fatty acids by weight) of the triacylglyceride fraction extracted from liver of pregnant rats fed different concentrations of oregano volatile oil.

Fatty Acid	*Diet*			
	Control	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	0.25±0.07	0.34±0.08	0.31±0.04	0.18±0.02
16:0	25.7±0.76	25.3±0.66	27.2±1.18	26.4±1.67
17:0	0.27±0.02	0.35±0.04	0.21±0.01	0.32±0.05
18:0	9.01±2.56	6.75±0.69[a]	4.86±0.92[b]	9.84±1.67
20:0	tr.	tr.	tr.	tr.
Total Saturates	***35.2***	***32.7***	***32.6***	***36.7***
14:1 n-5	tr.	tr.	tr.	tr.
16:1 n-7	0.96±0.28	1.90±0.3[c]	1.46±0.15[b]	2.09±0.69[c]
17:1 n-7	0.09±0.00	0.12±0.0	0.10±0.01	0.08±0.00
18:1 n-9	13.1±2.35	13.6±0.8	14.5±1.80	9.91±1.24
18:1 n-7	1.89±0.24	2.44±0.16	1.99±0.25	1.97±0.27
20:1 n-9	0.30±0.10	0.37±0.05	0.24±0.04	0.27±0.08
22:1 n-9	tr.	tr.	tr.	tr.
Total Monoenes	***16.3***	***18.4***	***18.3***	***14.3***
18:2 n-6	21.5±2.17	23.8±1.08	27.5±0.27	22.1±1.05
18:3 n-6	0.12±0.01	0.24±0.03[c]	0.18±0.02[b]	0.15±0.05
20:2 n-6	0.18±0.09	0.60±0.13[c]	0.19±0.04	0.45±0.11[c]
20:3 n-6	0.37±0.03	0.40±0.09	0.32±0.03	0.36±0.05
20:4 n-6	10.2±1.33	7.06±1.33	6.99±0.49	11.7±1.13
22:4 n-6	1.27±0.08	0.44±0.09[b]	0.59±0.08[b]	0.31±0.11[b]
Total n-6 PUFAs	***33.6***	***32.6***	***35.8***	***35.1***
18:3 n-3	1.14±0.18	1.39±0.18	1.74±0.22	1.62±0.13
18:4 n-3	0.06±0.02	tr.	tr.	tr.
20:5 n-3	0.76±0.13	0.63±0.08	1.45±0.21[a]	1.46±0.29[a]
22:3 n-3	tr.	tr.	tr.	tr.
22:5 n-3	3.34±0.61	2.25±0.30	2.95±0.45	1.85±0.12
22:6 n-3	14.63±0.92	6.84±1.27[b]	7.95±1.01[b]	10.93±1.69[a]
Total n-3 PUFAs	***19.9***	***11.2***	***14.1***	***15.9***
Total PUFA	***53.6***	***43.7***	***49.9***	***51.0***
U/S ratio[1]	***0.46***	***0.56***	***0.56***	***0.39***
P/S ratio[2]	***1.52***	***1.34***	***1.53***	***1.39***
n-6/n-3 ratio[3]	***1.69***	***2.91***	***2.54***	***2.21***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 26. Fatty acid composition (percentage of major fatty acids by weight) of the free fatty acid fraction extracted from liver of pregnant rats fed different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	0.26±0.08	0.37±0.04	0.43±0.11	0.30±0.06
16:0	26.7±1.74	24.0±1.75	23.7±1.89	25.6±0.80
17:0	0.26±0.04	0.30±0.04	0.36±0.16	0.26±0.04
18:0	16.72±2.88	9.48±0.98^{b}	8.25±1.54^{b}	11.10±1.59^{b}
20:0	tr.	tr.	tr.	tr.
Total Saturates	***43.9***	***34.2***	***21.8***	***37.2***
14:1 n-5	tr.	tr.	tr.	tr.
16:1 n-7	1.25±0.23	1.22±0.17	1.14±0.21	1.46±0.14
17:1 n-7	0.07±0.01	0.13±0.02^{c}	0.16±0.17^{c}	0.09±0.01
18:1 n-9	8.85±1.10	15.5±1.78^{b}	13.1±0.59	11.9±1.15
18:1 n-7	1.67±0.12	2.20±0.37	1.73±0.01	2.17±0.26
20:1 n-9	0.09±0.03	0.28±0.07	0.14±0.03	0.27±0.06
22:1 n-9	tr.	tr.	tr.	tr.
Total Monoenes	***11.9***	***19.3***	***16.4***	***15.9***
18:2 n-6	16.6±2.70	20.8±1.73	24.4±3.10	21.7±1.60
18:3 n-6	0.12±0.02	0.22±0.04^{a}	0.21±0.00^{a}	0.14±0.04
20:2 n-6	0.08±0.02	0.40±0.06^{c}	0.13±0.01	0.43±0.04^{c}
20:3 n-6	0.37±0.05	0.54±0.13	0.41±0.07	0.35±0.07
20:4 n-6	9.49±1.01	6.13±1.08	7.18±0.37	9.96±0.58
22:4 n-6	0.76±0.43	0.48±0.07	0.53±0.07	0.30±0.12
Total n-6 PUFAs	***27.4***	***28.5***	***32.9***	***32.8***
18:3 n-3	1.01±0.33	1.26±0.11	1.73±0.03^{b}	1.29±0.25
18:4 n-3	0.06±0.02	0.07±0.03	0.06±0.01	0.08±0.03
20:5 n-3	0.78±0.21	0.89±0.06	1.54±0.17^{c}	1.20±0.70
22:3 n-3	tr.	tr.	tr.	tr.
22:5 n-3	3.08±0.81	2.71±0.43	3.03±0.43	2.37±0.66
22:6 n-3	15.7±0.89	10.9±1.22^{a}	9.68±0.09^{b}	11.1±0.85^{a}
Total n-3 PUFAs	***20.7***	***15.8***	***16.0***	***16.0***
Total PUFA	***48.1***	***44.3***	***48.9***	***48.9***
U/S ratio[1]	***0.27***	***0.56***	***0.75***	***0.43***
P/S ratio[2]	***1.10***	***1.30***	***2.25***	***1.31***
n-6/n-3 ratio[3]	***1.32***	***1.80***	***2.06***	***2.05***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as ^{a} P<0.05; ^{b} P<0.01; ^{c} P<0.001. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 27. Fatty acid composition (percentage of major fatty acids by weight) of the phospholipid fraction extracted from liver of pregnant rats fed different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	0.06±0.00	0.09±0.03^{c}	0.08±0.01^{b}	0.09±0.03^{c}
16:0	21.9±0.87	19.1±1.35	21.8±0.61	21.3±1.04
17:0	0.33±0.01	0.47±0.05^{a}	0.30±0.02	0.36±0.01
18:0	23.2±0.71	27.1±1.65	22.5±0.71	24.6±1.58
20:0	tr.	tr.	tr.	tr.
Total Saturates	***45.6***	***46.8***	***44.7***	***46.4***
14:1 *n-5*	tr.	tr.	tr.	tr.
16:1 *n-7*	0.26±0.02	0.38±0.05^{a}	0.35±0.04^{a}	0.40±0.05^{a}
17:1 *n-7*	0.13±0.01	0.12±0.01	0.12±0.00	0.14±0.02
18:1 *n-9*	2.06±0.16	3.22±0.18^{c}	2.71±0.44^{a}	2.59±0.19^{a}
18:1 *n-7*	1.55±0.03	1.74±0.11	1.44±0.02^{a}	1.57±0.04
20:1 *n-9*	0.07±0.01	0.09±0.00	0.08±0.01	0.08±0.01
22:1 *n-9*	tr.	tr.	tr.	tr.
Total Monoenes	***4.07***	***5.55***	***4.70***	***4.78***
18:2 *n-6*	8.79±0.50	11.9±0.68^{b}	8.90±0.67	9.68±0.41
18:3 *n-6*	0.08±0.00	0.13±0.01^{c}	0.10±0.01^{a}	tr.
20:2 *n-6*	0.22±0.01	0.26±0.03	0.18±0.00	0.27±0.00^{a}
20:3 *n-6*	0.26±0.02	0.82±0.08^{c}	0.23±0.01	0.32±0.01
20:4 *n-6*	12.9±0.86	15.1±1.03	12.9±0.84	14.4±0.97
22:4 *n-6*	0.24±0.01	0.19±0.03	0.21±0.00^{b}	0.22±0.06
Total n-6 PUFAs	***22.5***	***28.4***	***22.5***	***24.9***
18:3 *n-3*	0.08±0.00	0.11±0.01^{b}	0.12±0.02^{a}	0.11±0.01^{b}
18:4 *n-3*	tr.	tr.	tr.	tr.
20:5 *n-3*	0.22±0.03	0.65±0.09^{c}	0.30±0.04	0.35±0.09
22:3 *n-3*	tr.	tr.	tr.	tr.
22:5 *n-3*	2.22±0.09	2.25±0.09	2.28±0.14	1.67±0.29^{a}
22:6 *n-3*	25.3±0.98	18.1±1.62^{b}	25.3±0.37	22.1±1.16
Total n-3 PUFAs	***27.8***	***21.1***	***27.9***	***24.2***
Total PUFA	***50.3***	***49.5***	***50.5***	***49.1***
U/S ratio[1]	***0.09***	***0.12***	***0.11***	***0.10***
P/S ratio[2]	***1.10***	***1.06***	***1.13***	***1.06***
n-6/n-3 ratio[3]	***0.81***	***1.35***	***0.81***	***1.03***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as ^{a} P<0.05; ^{b} P<0.01; ^{c} P<0.001. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 28. Fatty acid composition (percentage of major fatty acids by weight) of the phospholipid fraction extracted from brain of neonatal rats fed *in utero* different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	1.80±0.09	1.34±0.13[a]	1.39±0.10[b]	1.78±0.12
16:0	34.6±0.65	34.6±0.94	33.7±0.61	36.2±0.81
17:0	0.14±0.01	0.14±0.01	0.13±0.01	0.12±0.01
18:0	16.9±0.29	16.8±0.33	16.3±0.04	16.8±0.34
20:0	tr.	tr.	tr.	tr.
Total Saturates	***53.5***	***52.9***	***51.5***	***54.9***
14:1 *n-5*	tr.	tr.	tr.	tr.
16:1 *n-7*	2.02±0.19	1.74±0.10	1.88±0.04	2.02±0.24
17:1 *n-7*	1.19±0.06	1.24±0.09	0.55±0.29[b]	0.77±0.09[c]
18:1 *n-9*	13.04±0.26	11.97±0.26[b]	12.66±0.19	13.44±0.22
18:1 *n-7*	3.60±0.14	3.26±0.09[a]	3.49±0.15	3.65±0.22
20:1 *n-9*	0.25±0.01	0.23±0.02	0.21±0.02	0.27±0..02
22:1 *n-9*	tr.	tr.	0.14±0.00[a]	tr.
Total Monoenes	***20.1***	***18.5***	***18.9***	***20.2***
18:2 *n-6*	1.04±0.04	1.12±0.04	1.03±0.06	0.86±0.07[a]
18:3 *n-6*	0.10±0.00	0.13±0.01	0.11±0.01	0.09±0.01[a]
20:2 *n-6*	0.14±0.02	0.16±0.01	0.18±0.02	0.19±0.04
20:3 *n-6*	0.50±0.02	0.49±0.01	0.51±0.02	0.41±0.04[a]
20:4 *n-6*	11.3±0.26	12.4±0.42[a]	11.6±0.12	11.2±0.34
22:4 *n-6*	2.24±0.05	2.47±0.12	2.10±0.05	2.04±0.11
Total n-6 PUFAs	***15.4***	***16.8***	***15.5***	***14.8***
18:3 *n-3*	0.09±0.00	tr.	tr.	0.12±0.04
18:4 *n-3*	tr.	tr.	0.14±0.04	tr.
20:5 *n-3*	0.13±0.02	0.09±0.01	0.13±0.01	0.11±0.34
22:3 *n-3*	1.09±0.04	tr.[c]	tr.[c]	1.21±0.14
22:5 *n-3*	0.33±0.01	0.34±0.01	0.42±0.04	0.24±0.03
22:6 *n-3*	11.1±0.30	12.2±0.45	12.5±0.35[a]	10.8±0.73
Total n-3 PUFAs	***12.7***	***12.6***	***13.2***	***12.5***
Total PUFA	***28.1***	***29.5***	***28.7***	***27.3***
U/S ratio[1]	***0.38***	***0.35***	***0.37***	***0.37***
P/S ratio[2]	***0.52***	***0.56***	***0.56***	***0.50***
n-6/n-3 ratio[3]	***1.21***	***1.33***	***1.17***	***1.18***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] P<0.05; [b] P<0.01; [c] P<0.001. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 29. Fatty acid composition (percentage of major acids by weight) of the cholesteryl ester fraction from heart of neonatal rats fed *in utero* different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	3.78±0.10	0.70±0.02^{c}	2.45±1.23	tr.^{c}
16:0	23.3±1.33	21.5±0.98	19.9±1.16	24.7±1.77
17:0	0.93±0.18	0.66±0.13	1.34±0.52	0.51±0.01
18:0	13.2±0.59	9.69±0.79^{b}	9.02±1.58^{b}	10.9±1.10
20:0	1.79±0.19	tr.^{c}	tr.^{c}	tr.^{c}
Total Saturates	***42.9***	***32.6***	***32.7***	***36.2***
14:1 *n-5*	tr.	tr.	tr.	tr.
16:1 *n-7*	4.73±0.24	2.86±0.54^{b}	4.72±0.80	0.98±0.02^{c}
17:1 *n-7*	0.56±0.22	0.43±0.08	tr.^{c}	tr.^{c}
18:1 *n-9*	15.6±0.52	17.0±2.22	16.7±1.80	29.7±3.15^{c}
18:1 *n-7*	2.36±0.20	3.17±0.44	2.29±0.21	3.55±0.07^{b}
20:1 *n-9*	0.46±0.06	0.40±0.05	0.56±0.05	0.69±0.02
22:1 *n-9*	tr.	tr.	tr.	tr.
Total Monoenes	***23.7***	***23.9***	***24.3***	***34.9***
18:2 *n-6*	10.3±0.92	13.4±0.83^{a}	6.14±0.18^{b}	14.7±1.94^{a}
18:3 *n-6*	2.26±0.05	tr.^{c}	1.83±0.72	0.40±0.00^{c}
20:2 *n-6*	2.87±0.26	3.78±0.52	6.22±0.99^{c}	0.69±0.11^{b}
20:3 *n-6*	1.43±0.10	1.01±0.12	tr.^{c}	0.50±0.00^{b}
20:4 *n-6*	13.8±1.42	20.5±1.16^{b}	9.85±1.03	14.4±0.90
22:4 *n-6*	2.20±0.60	1.43±0.18	tr.^{c}	0.77±0.03
Total n-6 PUFAs	***32.8***	***40.1***	***24.0***	***31.4***
18:3 *n-3*	1.69±0.32	0.55±0.07	tr.^{c}	tr.^{c}
18:4 *n-3*	0.62±0.09	1.33±0.11^{b}	3.06±0.41^{c}	tr.^{c}
20:5 *n-3*	2.53±0.47	1.66±0.61	tr.^{c}	tr.^{c}
22:3 *n-3*	0.27±0.01	tr.	tr.	tr.
22:5 *n-3*	1.39±0.42	1.76±0.11	tr.^{c}	tr.^{c}
22:6 *n-3*	2.73±0.32	4.78±0.70^{a}	5.04±0.57^{a}	1.62±0.02
Total n-3 PUFAs	***9.23***	***10.1***	***8.10***	***1.62***
Total PUFA	***42.1***	***50.2***	***32.1***	***33.1***
U/S ratio[1]	***0.55***	***0.73***	***0.74***	***0.96***
P/S ratio[2]	***0.98***	***1.54***	***0.98***	***0.91***
n-6/n-3 ratio[3]	***3.55***	***3.98***	***2.96***	***1.94***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as ^{a} P<0.05; ^{b} P<0.01; ^{c} P<0.001. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 30. Fatty acid composition (percentage of major acids by weight) of the triacylglyceride fraction from heart of neonatal rats fed *in utero* different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	0.49±0.17	tr.[c]	tr.[c]	tr.[c]
14:0	1.59±0.15	1.66±0.35	1.14±0.10	tr.[c]
16:0	25.7±2.55	26.7±1.33	25.6±0.16	25.9±2.41
17:0	0.61±0.05	0.46±0.03[a]	0.97±0.23[a]	0.50±0.00
18:0	10.8±0.43	7.01±0.46[c]	9.09±0.35[a]	10.6±1.20
20:0	0.20±0.42	0.22±0.09	0.40±0.20	0.21±0.02
Total Saturates	***32.4***	***36.1***	***37.2***	***37.3***
14:1 *n-5*	0.46±0.16	tr.[b]	tr.[b]	tr.[b]
16:1 *n-7*	1.84±0.23	1.31±0.32	1.79±0.46	2.82±0.07[a]
17:1 *n-7*	0.29±0.11	0.31±0.02	tr.	tr.
18:1 *n-9*	27.7±3.10	25.3±2.88	36.8±1.68[a]	27.4±4.80
18:1 *n-7*	3.04±0.17	2.93±0.17	2.58±0.22	3.16±0.70
20:1 *n-9*	0.52±0.01	0.60±0.05	0.70±0.16[a]	0.49±0.01
22:1 *n-9*	0.08±0.00	0.06±0.00[a]	tr.	tr.
Total Monoenes	***33.9***	***30.5***	***41.9***	***33.9***
18:2 *n-6*	15.4±1.01	19.3±1.16[a]	16.1±1.79	12.8±1.50
18:3 *n-6*	0.46±0.08	0.53±0.10	tr.	tr.
20:2 *n-6*	1.77±0.18	0.98±0.11[c]	1.68±0.18	3.95±0.55
20:3 *n-6*	0.45±0.05	0.94±0.11[b]	tr.	tr.
20:4 *n-6*	3.08±0.13	2.71±0.19	1.63±0.28[c]	3.95±1.20[c]
22:4 *n-6*	0.51±0.13	0.73±0.10	tr.[c]	1.40±0.16[c]
Total n-6 PUFAs	***21.6***	***25.2***	***19.4***	***22.1***
18:3 *n-3*	0.70±0.08	0.93±0.16	1.00±0.05	tr.[c]
18:4 *n-3*	0.80±0.11	tr.[c]	1.49±0.24	tr.[c]
20:5 *n-3*	0.51±0.13	0.52±0.08	tr.[c]	tr.[c]
22:3 *n-3*	0.33±0.04	tr.[c]	tr.[c]	tr.[c]
22:5 *n-3*	0.73±0.02	1.31±0.16[c]	0.84±0.15	0.50±0.01[b]
22:6 *n-3*	2.04±0.30	2.91±0.33	3.06±0.10	2.90±0.74
Total n-3 PUFAs	***5.11***	***5.72***	***6.39***	***3.40***
Total PUFA	***26.8***	***30.9***	***25.8***	***25.5***
U/S ratio[1]	***1.05***	***0.85***	***1.13***	***0.91***
P/S ratio[2]	***0.83***	***0.86***	***0.69***	***0.69***
n-6/n-3 ratio[3]	***4.23***	***4.41***	***3.04***	***6.50***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 31. Fatty acid composition (percentage of major acids by weight) of the free fatty acid fraction from heart of neonatal rats fed *in utero* different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	0.11±0.00	0.07±0.01	tr.[b]	tr.[b]
14:0	0.70±0.10	0.87±0.18	0.39±0.07[a]	0.30±0.02[b]
16:0	15.3±0.25	15.9±1.00	14.2±0.59	13.9±1.01
17:0	0.40±0.03	0.36±0.04	0.28±0.01	0.31±0.01
18:0	12.1±0.20	13.6±0.45[b]	10.7±0.42[b]	11.3±0.04[b]
20:0	0.12±0.02	0.13±0.01	0.13±0.00	0.10±0.01
Total Saturates	***28.8***	***31.0***	***25.7***	***25.9***
14:1 *n-5*	0.21±0.09	tr.[b]	tr.[b]	tr.[b]
16:1 *n-7*	1.24±0.17	0.77±0.11[a]	0.68±0.03[a]	1.04±0.29
17:1 *n-7*	0.20±0.01	0.24±0.02	0.19±0.02	0.22±0.03
18:1 *n-9*	13.7±0.15	13.7±0.51	13.9±0.54	14.9±0.42[b]
18:1 *n-7*	3.28±0.08	3.61±0.10[a]	3.49±0.14	3.60±0.18
20:1 *n-9*	0.43±0.03	0.52±0.04	0.46±0.03	0.50±0.03
22:1 *n-9*	0.09±0.01	0.13±0.01[b]	0.12±0.01	0.10±0.00
Total Monoenes	***19.2***	***19.0***	***18.9***	***20.4***
18:2 *n-6*	14.1±0.27	15.0±1.19	13.6±0.28	14.7±0.75
18:3 *n-6*	0.47±0.06	0.36±0.06	0.31±0.02	0.58±0.02[b]
20:2 *n-6*	3.57±0.20	1.58±0.18[c]	3.04±0.13	1.98±0.14[c]
20:3 *n-6*	1.62±0.06	1.91±0.07[b]	1.93±0.11[a]	1.88±0.11[a]
20:4 *n-6*	18.2±0.93	20.2±0.71	20.3±0.36	20.7±0.38
22:4 *n-6*	1.88±0.08	2.39±0.11[a]	2.09±0.05	2.19±0.18
Total n-6 PUFAs	***39.8***	***41.4***	***41.2***	***42.2***
18:3 *n-3*	1.95±0.20	0.52±0.06[a]	0.35±0.00[b]	0.59±0.12[c]
18:4 *n-3*	0.16±0.06	0.04±0.00	0.04±0.00	0.23±0.18
20:5 *n-3*	1.00±0.07	1.12±0.07	0.94±0.44	1.36±0.07[b]
22:3 *n-3*	0.32±0.03	tr.[c]	tr.[c]	0.40±0.05
22:5 *n-3*	2.14±0.09	3.15±0.13[c]	2.74±0.13[b]	2.39±0.24
22:6 *n-3*	6.74±0.87	9.26±0.54[b]	9.15±0.43[b]	8.05±0.63[a]
Total n-3 PUFAs	***12.3***	***14.1***	***13.2***	***13.0***
Total PUFA	***52.1***	***55.5***	***54.5***	***55.2***
U/S ratio[1]	***0.67***	***0.61***	***0.74***	***0.79***
P/S ratio[2]	***1.81***	***1.79***	***2.12***	***2.13***
n-6/n-3 ratio[3]	***3.24***	***2.94***	***3.12***	***3.25***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 32. Fatty acid composition (percentage of major acids by weight) of the phospholipid fraction from heart of neonatal rats fed *in utero* different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	0.47±0.03	0.40±0.08	0.32±0.08	tr.
16:0	25.6±0.56	23.8±1.19	24.7±0.53	25.9±2.41
17:0	0.40±0.01	0.37±0.03	0.34±0.02[b]	0.38±0.01
18:0	21.2±0.36	21.2±0.48	21.3±0.02	21.9±0.45
20:0	0.32±0.04	0.33±0.06	0.38±0.04	0.29±0.01
Total Saturates	***47.9***	***46.1***	***46.9***	***48.6***
14:1 *n-5*	0.23±0.03	tr.[c]	tr.[c]	tr.[c]
16:1 *n-7*	0.67±0.07	0.51±0.06	0.43±0.02	0.87±0.16
17:1 *n-7*	0.49±0.08	0.35±0.08	0.59±0.15	0.55±0.09
18:1 *n-9*	*12.3±0.19*	*9.72±1.10*[b]	*13.2±0.84*	*16.1±0.38*[c]
18:1 *n-7*	4.39±0.07	4.36±0.09	4.43±0.12	4.97±0.41[a]
20:1 *n-9*	0.39±0.05	0.36±0.10	0.27±0.01	0.33±0.13
22:1 *n-9*	0.10±0.00	0.12±0.05	0.08±0.01[b]	0.42±0.29
Total Monoenes	***18.6***	***15.4***	***19.0***	***23.2***
18:2 *n-6*	7.68±0.16	7.19±0.17[a]	7.36±0.36	8.00±0.19
18:3 *n-6*	0.25±0.02	0.14±0.02[b]	0.18±0.01[a]	0.27±0.07
20:2 *n-6*	0.71±0.06	0.55±0.04	0.52±0.04	0.64±0.02
20:3 *n-6*	1.01±0.03	1.01±0.06	1.08±0.07	0.99±0.07
20:4 *n-6*	16.5±0.33	22.0±0.44[c]	16.5±0.68	16.4±0.31
22:4 *n-6*	1.48±0.04	1.83±0.15[b]	1.51±0.07	1.40±0.16
Total n-6 PUFAs	***27.7***	***32.7***	***27.2***	***27.7***
18:3 *n-3*	0.13±0.02	0.11±0.02	0.08±0.02	tr.[b]
18:4 *n-3*	0.09±0.00	tr.[b]	tr.	tr.
20:5 *n-3*	0.57±0.06	0.35±0.02[a]	0.65±0.09	0.56±0.09
22:3 *n-3*	0.34±0.14	tr.	tr.	tr.
22:5 *n-3*	1.37±0.05	0.21±0.02[c]	1.54±0.10	1.18±0.18
22:6 *n-3*	4.25±0.15	1.91±0.15[c]	4.86±0.26	3.32±0.64[a]
Total n-3 PUFAs	***6.75***	***2.61***	***7.13***	***5.06***
Total PUFA	***34.4***	***35.3***	***34.3***	***32.8***
U/S ratio[1]	***0.39***	***0.33***	***0.41***	***0.48***
P/S ratio[2]	***0.72***	***0.77***	***0.73***	***0.68***
n-6/n-3 ratio[3]	***4.10***	***12.53***	***3.82***	***5.47***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 33. Fatty acid composition (percentage of major acids by weight) of the cholesteryl ester fraction from liver of neonatal rats fed in utero different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	0.07±0.01	tr.	tr.
14:0	0.58±0.06	0.66±0.07	0.56±0.28	0.58±0.06
16:0	13.4±0.67	15.4±0.55[a]	16.1±1.71	15.7±1.31
17:0	0.35±0.01	0.41±0.02[a]	0.51±0.08[b]	0.46±0.02[c]
18:0	4.26±0.07	4.33±0.23	4.21±0.21	5.41±0.40[b]
20:0	0.10±0.00	0.11±0.02	tr.[b]	tr.[b]
Total Saturates	***18.7***	***20.9***	***21.4***	***22.2***
14:1 *n-5*	0.13±0.02	0.08±0.00	0.27±0.02[b]	tr.
16:1 *n-7*	1.58±0.24	1.65±0.31	1.63±0.08	1.72±0.25
17:1 *n-7*	0.30±0.01	0.34±0.03	0.41±0.08[a]	0.51±0.19[a]
18:1 *n-9*	19.4±0.47	22.1±0.98[a]	22.9±2.54	20.4±0.85
18:1 *n-7*	2.35±0.07	2.05±0.11[a]	2.36±0.09	2.97±0.45[a]
20:1 *n-9*	0.34±0.02	0.18±0.03[c]	tr.	0.26±0.01
22:1 *n-9*	0.11±0.01	tr.	tr.	tr.
Total Monoenes	***24.2***	***26.4***	***27.6***	***27.9***
18:2 *n-6*	18.5±0.74	17.5±1.02	17.1±1.35	12.7±1.30[c]
18:3 *n-6*	0.70±0.03	0.62±0.03	0.79±0.26	0.45±0.04[c]
20:2 *n-6*	0.60±0.04	0.82±0.08[a]	1.36±0.11[c]	0.44±0.04
20:3 *n-6*	1.02±0.08	0.91±0.05	1.23±0.08	1.17±0.10
20:4 *n-6*	18.6±0.67	15.6±0.53[b]	21.1±1.85	12.1±2.08[c]
22:4 *n-6*	1.50±0.08	1.34±0.10	1.58±0.08	1.21±0.23
Total n-6 PUFAs	***40.9***	***36.7***	***43.1***	***40.1***
18:3 *n-3*	0.15±0.03	0.81±0.10[c]	0.54±0.02[c]	0.36±0.20
18:4 *n-3*	0.41±0.08	0.31±0.05	0.49±0.21	0.43±0.04
20:5 *n-3*	3.30±0.16	3.37±0.29	3.71±0.49	2.87±0.55
22:3 *n-3*	0.23±0.02	tr.[c]	tr.[c]	0.29±0.04
22:5 *n-3*	2.86±0.06	3.29±0.17[a]	3.19±0.31	0.85±0.05[c]
22:6 *n-3*	9.47±0.47	9.68±0.54	9.30±3.24	6.74±1.24[b]
Total n-3 PUFAs	***16.4***	***17.5***	***17.2***	***11.5***
Total PUFA	***57.3***	***54.2***	***60.4***	***51.6***
U/S ratio[1]	***1.29***	***1.26***	***1.29***	***1.26***
P/S ratio[2]	***3.06***	***2.59***	***2.82***	***2.32***
n-6/n-3 ratio[3]	***2.49***	***2.10***	***2.51***	***3.49***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] P<0.05; [b] P<0.01; [c] P<0.001. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 34. Fatty acid composition (percentage of major acids by weight) of the triacylglyceride fraction from liver of neonatal rats fed *in utero* different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	0.10±0.02	0.19±0.01[a]	0.06±0.03	0.15±0.05
14:0	0.89±0.15	1.26±0.18	0.84±0.11	1.46±0.03[a]
16:0	46.2±3.45	42.5±1.96	45.9±2.28	48.0±2.56
17:0	0.47±0.05	0.42±0.07	0.34±0.03[a]	0.44±0.03
18:0	3.62±0.35	3.52±0.08	3.84±0.36	4.94±0.28[a]
20:0	0.06±0.01	0.07±0.02	0.20±0.04[b]	0.08±0.01
Total Saturates	***51.4***	***48.0***	***51.3***	***55.1***
14:1 *n-5*	tr.	tr.	tr.	tr.
16:1 *n-7*	0.91±0.10	1.38±0.15[a]	1.53±0.18[b]	1.58±0.20[b]
17:1 *n-7*	0.13±0.03	0.08±0.03	0.14±0.04	0.16±0.03
18:1 *n-9*	14.9±0.50	15.9±0.55	14.6±0.95	19.0±0.69[c]
18:1 *n-7*	2.21±0.14	2.32±0.12	2.28±0.33	3.13±0.19[b]
20:1 *n-9*	0.43±0.06	0.33±0.04	0.28±0.04	0.54±0.05
22:1 *n-9*	tr.	tr.	tr.	0.06±0.01
Total Monoenes	***18.6***	***20.1***	***18.8***	***24.5***
18:2 *n-6*	11.5±0.47	14.2±1.44	11.0±0.63	10.9±3.59
18:3 *n-6*	0.52±0.03	0.56±0.05	0.51±0.07	0.55±0.06
20:2 *n-6*	0.43±0.05	0.42±0.03	0.27±0.06	0.47±0.14
20:3 *n-6*	0.67±0.08	0.97±0.11	0.71±0.05	1.29±0.09[b]
20:4 *n-6*	3.76±0.31	4.48±0.20	4.36±0.12	4.22±0.66
22:4 *n-6*	1.43±0.07	1.61±0.06	1.49±0.14	1.87±0.29
Total n-6 PUFAs	***18.3***	***22.2***	***18.4***	***19.4***
18:3 *n-3*	0.61±0.07	0.48±0.06	0.46±0.08	0.57±0.03
18:4 *n-3*	0.08±0.00	tr.[a]	tr.[a]	0.06±0.01
20:5 *n-3*	1.25±0.10	1.06±0.11	1.38±0.11	1.09±0.05
22:3 *n-3*	0.24±0.04	tr.[b]	tr.[b]	0.32±0.02
22:5 *n-3*	1.91±0.16	2.83±0.22[b]	2.27±0.13	2.81±0.76[a]
22:6 *n-3*	6.26±0.73	9.01±0.51[b]	8.29±0.40[a]	5.55±1.32
Total n-3 PUFAs	***10.4***	***13.4***	***12.4***	***10.4***
Total PUFA	***28.6***	***35.6***	***30.8***	***29.8***
U/S ratio[1]	***0.36***	***0.42***	***0.37***	***0.45***
P/S ratio[2]	***0.56***	***0.74***	***0.60***	***0.54***
n-6/n-3 ratio[3]	***1.76***	***1.66***	***1.48***	***1.87***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 35. Fatty acid composition (percentage of major acids by weight) of the free fatty acid fraction from liver of neonatal rats fed *in utero* different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	0.17±0.01	0.49±0.07[c]	0.07±.0.01[c]	0.14±0.03
14:0	0.63±0.02	1.49±0.15[c]	0.47±0.03[b]	0.59±0.04
16:0	21.7±0.77	19.9±0.59	21.3±0.69	21.1±0.62
17:0	0.32±0.02	0.24±0.01[c]	0.27±0.02[a]	0.31±0.01
18:0	8.17±0.49	6.57±0.62	8.26±0.50	9.27±0.41
20:0	tr.	tr.	tr.	tr.
Total Saturates	***31.0***	***28.8***	***30.4***	***31.4***
14:1 *n-5*	tr.	tr.	tr.	tr.
16:1 *n-7*	1.47±0.06	1.20±0.08[b]	1.74±0.04[a]	1.51±0.13
17:1 *n-7*	0.13±0.01	0.13±0.03	0.12±0.02	0.12±0.01
18:1 *n-9*	13.5±0.24	*11.1±0.62[b]*	*11.8±0.21[b]*	*12.8±0.28*
18:1 *n-7*	2.42±0.11	1.95±0.07[b]	2.39±0.18	2.53±0.18
20:1 *n-9*	0.29±0.02	0.19±0.03[b]	0.22±0.01[a]	0.29±0.01
22:1 *n-9*	tr.	tr.	tr.	tr.
Total Monoenes	***17.9***	***14.6***	***16.3***	***17.3***
18:2 *n-6*	17.6±0.44	17.9±1.27	14.4±0.09[c]	17.7±0.87
18:3 *n-6*	0.68±0.04	0.53±0.03[a]	0.52±0.02[a]	0.50±0.05[a]
20:2 *n-6*	0.64±0.05	0.39±0.05[b]	0.41±0.07[a]	0.65±0.05
20:3 *n-6*	1.24±0.07	1.34±0.08	1.08±0.05	1.29±0.12
20:4 *n-6*	14.2±0.42	13.3±0.42	14.7±0.35	15.7±0.58[a]
22:4 *n-6*	1.64±0.06	1.54±0.06	1.42±0.06	1.72±0.18
Total n-6 PUFAs	***35.9***	***35.0***	***32.6***	***37.5***
18:3 *n-3*	0.29±0.07	0.51±0.04[a]	0.27±0.09	0.25±0.05
18:4 *n-3*	0.23±0.06	tr.[a]	tr.[a]	tr.[a]
20:5 *n-3*	1.91±0.09	2.24±0.38	1.89±0.13	1.84±0.09
22:3 *n-3*	0.30±0.02	tr.[c]	tr.[c]	0.35±0.06
22:5 *n-3*	2.91±0.16	3.81±0.19[b]	2.99±0.31	2.85±0.34
22:6 *n-3*	13.3±1.04	16.9±1.15[a]	15.3±0.36	13.0±0.34
Total n-3 PUFAs	***18.9***	***23.5***	***20.5***	***18.4***
Total PUFA	***54.9***	***58.6***	***53.0***	***55.9***
U/S ratio[1]	***0.58***	***0.51***	***0.54***	***0.55***
P/S ratio[2]	***1.77***	***2.03***	***1.74***	***1.78***
n-6/n-3 ratio[3]	***1.90***	***1.49***	***1.59***	***2.04***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] P<0.05; [b] P<0.01; [c] P<0.001. tr.:<0.05% weight. PUFAs: polyunsaturated fatty acids.[1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 36. Fatty acid composition (percentage of major acids by weight) of the phospholipid fraction from liver of neonatal rats fed *in utero* different concentrations of oregano volatile oil.

	Diet			
Fatty Acid	*Control*	*167mg Kg^{-1}*	*334mg Kg^{-1}*	*834mg Kg^{-1}*
12:0	tr.	tr.	tr.	tr.
14:0	0.49±0.01	0.62±0.10	0.61±0.10	0.54±0.08
16:0	32.9±0.76	27.2±0.50^{c}	36.3±1.17^{a}	33.2±0.49
17:0	0.50±0.04	0.44±0.04	0.44±0.05	0.44±0.02
18:0	19.8±0.55	18.6±0.67	20.1±2.42	19.7±0.30
20:0	0.18±0.01	0.06±0.01^{c}	0.12±0.06	0.14±0.01
Total Saturates	***53.9***	***46.9***	***57.5***	***53.9***
14:1 *n-5*	tr.	tr.	tr.	tr.
16:1 *n-7*	1.04±0.11	0.70±0.10^{a}	1.32±0.01	0.67±0.08
17:1 *n-7*	0.26±0.02	0.17±0.04^{a}	0.19±0.04^{a}	0.11±0.01^{c}
18:1 *n-9*	7.46±0.42	6.03±0.48^{a}	7.14±0.26	7.45±0.64
18:1 *n-7*	2.96±0.14	2.66±0.04^{a}	2.98±0.22	2.97±0.24
20:1 *n-9*	0.19±0.02	0.16±0.01	0.15±0.01	0.20±0.01
22:1 *n-9*	0.07±0.00	tr.^{b}	tr.^{c}	0.07±0.01
Total Monoenes	***9.98***	***9.76***	***11.8***	***11.5***
18:2 *n-6*	9.11±0.14	10.0±0.51	8.52±0.31	9.70±0.10^{a}
18:3 *n-6*	0.30±0.02	0.27±0.02	0.26±0.02	0.27±0.02
20:2 *n-6*	0.30±0.02	0.25±0.05	0.22±0.0^{a}	0.32±0.02
20:3 *n-6*	0.61±0.04	0.66±0.04	0.61±0.09	0.65±0.01
20:4 *n-6*	13.6±0.30	14.6±0.19^{b}	11.9±0.40^{b}	12.3±0.38
22:4 *n-6*	0.62±0.05	0.62±0.02	0.52±0.03	0.65±0.03
Total n-6 PUFAs	***24.6***	***26.4***	***22.0***	***23.8***
18:3 *n-3*	0.06±0.01	0.08±0.02	0.05±0.03	0.08±0.02
18:4 *n-3*	0.08±0.01	tr.	tr.	tr.
20:5 *n-3*	0.30±0.03	0.31±0.02	0.39±0.03	0.33±0.01
22:3 *n-3*	0.15±0.01	tr.^{c}	tr.^{c}	0.17±0.00
22:5 *n-3*	1.15±0.01	1.67±0.11^{b}	1.07±0.14	1.19±0.13
22:6 *n-3*	9.57±0.31	13.9±0.45^{c}	9.19±1.02	8.67±0.82
Total n-3 PUFAs	***11.31***	***16.05***	***10.70***	***10.44***
Total PUFA	***35.9***	***42.4***	***32.7***	***34.3***
U/S ratio[1]	***0.19***	***0.21***	***0.21***	***0.21***
P/S ratio[2]	***0.67***	***0.91***	***0.57***	***0.64***
n-6/n-3 ratio[3]	***2.18***	***1.64***	***2.06***	***2.29***

The results are expressed as percentage composition and are means ± S.E.M. (n=6). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as ^{a} P<0.05; ^{b} P<0.01; ^{c} P<0.001. tr.:<0.05% weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 37. Fatty acid composition (percentage of major acids by weight) of the triacylglyceride fraction from adipose of pregnant rats fed 50μg g^{-1} body weight clove or nutmeg volatile oils.

		Clove	*Nutmeg*
Fatty Acid	*Control*	*50μg g^{-1}*	*50μg g^{-1}*
12:0	0.14±0.01	0.09±0.01[c]	0.09±0.00[c]
14:0	1.31±0.05	1.04±0.07[a]	1.20±0.05
16:0	24.6±0.34	23.3±0.69	25.4±0.33
17:0	0.30±0.02	0.32±0.03	0.30±0.01
18:0	3.89±0.12	3.89±0.15	3.99±0.17
20:0	tr.	0.19±0.00[c]	0.08±0.01
Total Saturates	***30.2***	***28.8***	***30.0***
14:1 *n-5*	tr.	tr.	tr.
16:1 *n-7*	4.03±0.29	3.28±0.33	3.37±0.38
17:1 *n-7*	0.26±0.01	0.28±0.01	tr.[c]
18:1 *n-9*	27.7±0.39	27.7±0.43	27.2±0.51
18:1 *n-7*	2.78±0.06	2.90±0.08	3.04±0.05[b]
20:1 *n-9*	0.54±0.02	0.59±0.04	0.35±0.03[c]
22:1 *n-9*	tr.	tr.	tr.
Total Monoenes	***35.3***	***34.8***	***34.0***
18:2 *n-6*	30.7±0.89	33.4±0.61[a]	32.8±0.93
18:3 *n-6*	0.24±0.02	0.16±0.03	0.10±0.03[b]
20:2 *n-6*	0.25±0.01	0.28±0.02	tr.[c]
20:3 *n-6*	0.19±0.01	0.21±0.02	0.21±0.00
20:4 *n-6*	0.64±0.03	0.66±0.04	tr.[c]
22:4 *n-6*	tr.	tr.	0.19±0.02[c]
Total n-6 PUFAs	***32.1***	***34.7***	***33.3***
18:3 *n-3*	tr.	tr.	tr.
18:4 *n-3*	tr.	2.52±0.04[c]	tr.
20:5 *n-3*	0.19±0.01	0.23±0.03	tr.[c]
22:3 *n-3*	tr.	tr.	tr.
22:5 *n-3*	1.04±0.09	0.51±0.01[c]	0.45±0.05[b]
22:6 *n-3*	tr.	1.11±0.13[c]	0.99±0.11[c]
Total n-3 PUFAs	***1.37***	***4.46***	***1.52***
Total PUFA	***33.4***	***39.2***	***34.9***
U/S ratio[1]	***1.17***	***1.21***	***1.13***
P/S ratio[2]	***1.11***	***1.36***	***1.13***
n-6/n-3 ratio[3]	***23.4***	***7.78***	***21.9***

The results are expressed as percentage composition and are means ± S.E.M. (n=5). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05% weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 38. Fatty acid composition (percentage of major acids by weight) of the phospholipid fraction from brain of pregnant rats fed 50μg g^{-1} body weight clove or nutmeg volatile oils.

		Clove	*Nutmeg*
Fatty Acid	*Control*	*50μg g^{-1}*	*50μg g^{-1}*
12:0	tr.	tr.	tr.
14:0	0.08±0.00	0.14±0.02[c]	0.10±0.01[b]
16:0	19.1±0.40	24.9±0.67[c]	21.6±0.34[c]
17:0	0.19±0.01	0.23±0.01[b]	0.23±0.01[b]
18:0	20.2±0.34	0.94±0.0[c]	20.9±0.35
20:0	0.73±0.10	0.67±0.06	0.45±0.04[b]
Total Saturates	***40.3***	***26.9***	***43.2***
14:1 *n-5*	tr.	tr.	tr.
16:1 *n-7*	0.28±0.01	0.38±0.01[c]	0.46±0.05[c]
17:1 *n-7*	0.27±0.05	tr.[b]	0.13±0.01[a]
18:1 *n-9*	20.4±0.46	26.4±0.79[c]	20.6±0.52
18:1 *n-7*	4.30±0.16	5.38±0.36[a]	4.26±0.13
20:1 *n-9*	2.90±0.27	3.69±0.29[a]	2.35±0.05
22:1 *n-9*	0.22±0.04	0.19±0.01	0.17±0.01
Total Monoenes	***28.3***	***36.1***	***27.9***
18:2 *n-6*	0.73±0.06	1.06±0.0[a]	0.72±0.02
18:3 *n-6*	0.06±0.00	tr.	tr.
20:2 *n-6*	0.22±0.03	0.12±0.01	0.16±0.01
20:3 *n-6*	0.47±0.01	0.56±0.04[a]	0.37±0.02[b]
20:4 *n-6*	9.52±0.36	12.4±0.16[c]	9.42±0.38
22:4 *n-6*	3.43±0.09	tr.	2.91±0.16[a]
Total n-6 PUFAs	***14.4***	***14.2***	***13.6***
18:3 *n-3*	tr.	tr.	0.16±0.02
18:4 *n-3*	tr.	0.11±0.01[a]	tr.
20:5 *n-3*	0.53±0.01	1.51±1.00	0.35±0.09
22:3 *n-3*	0.16±0.04	0.30±0.01[c]	tr.[c]
22:5 *n-3*	0.52±0.31	0.25±0.00	0.69±0.06
22:6 *n-3*	17.0±0.10	20.6±0.70[c]	14.1±0.69[b]
Total n-3 PUFAs	***18.2***	***22.7***	***15.3***
Total PUFA	***32.7***	***36.9***	***28.8***
U/S ratio[1]	***0.70***	***1.34***	***0.65***
P/S ratio[2]	***0.81***	***1.37***	***0.67***
n-6/n-3 ratio[3]	***0.79***	***0.63***	***0.89***

The results are expressed as percentage composition and are means ± S.E.M. (n=5). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 39. Fatty acid composition (percentage of major acids by weight) of the triacylglyceride fraction from heart of pregnant rats fed 50μg g^{-1} body weight clove or nutmeg volatile oils.

		Clove	*Nutmeg*
Fatty Acid	*Control*	*50μg g^{-1}*	*50μg g^{-1}*
12:0	tr.	tr.	tr.
14:0	1.30±0.08	1.15±0.08	1.12±0.07
16:0	26.0±0.64	23.4±0.67[a]	26.5±0.51
17:0	0.37±0.02	0.43±0.12	0.35±0.03
18:0	7.12±0.18	7.85±1.03	6.71±0.28
20:0	0.14±0.01	0.21±0.08	tr.[c]
Total Saturates	***34.9***	***33.0***	***34.7***
14:1 *n-5*	tr.	tr.	tr.
16:1 *n-7*	3.75±0.34	5.05±0.17[a]	3.26±0.12
17:1 *n-7*	0.33±0.03	0.22±0.02[a]	0.27±0.02
18:1 *n-9*	28.1±0.97	30.3±0.28	28.8±0.65
18:1 *n-7*	3.05±0.10	3.25±0.11	3.45±0.24
20:1 *n-9*	0.53±0.12	0.68±0.12	0.62±0.04
22:1 *n-9*	tr.	tr.	tr.
Total Monoenes	***35.8***	***39.5***	***36.4***
18:2 *n-6*	24.8±0.93	25.9±1.21	25.3±0.62
18:3 *n-6*	0.12±0.02	0.09±0.00	0.15±0.06
20:2 *n-6*	0.34±0.02	0.64±0.06[c]	0.37±0.06
20:3 *n-6*	0.19±0.02	0.11±0.02	0.16±0.02
20:4 *n-6*	1.51±0.09	0.90±0.23[a]	0.95±0.08[b]
22:4 *n-6*	0.25±0.02	tr.	0.19±0.01
Total n-6 PUFAs	***27.2***	***27.6***	***27.1***
18:3 *n-3*	0.07±0.03	0.07±0.02	1.34±0.07[c]
18:4 *n-3*	1.38±0.08	1.43±0.13	tr.[c]
20:5 *n-3*	0.09±0.00	0.05±0.00[a]	tr.[a]
22:3 *n-3*	tr.	tr.	tr.
22:5 *n-3*	0.34±0.02	0.27±0.12	0.30±0.08
22:6 *n-3*	1.02±0.01	0.87±0.10	0.98±0.11
Total n-3 PUFAs	***2.90***	***2.69***	***2.62***
Total PUFA	***30.1***	***30.3***	***29.8***
U/S ratio[1]	***1.03***	***1.20***	***1.05***
P/S ratio[2]	***0.86***	***0.92***	***0.86***
n-6/n-3 ratio[3]	***9.38***	***10.3***	***10.3***

The results are expressed as percentage composition and are means ± S.E.M. (n=5). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] P<0.05; [b] P<0.01; [c] P<0.001. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 40. Fatty acid composition (percentage of major acids by weight) of the free fatty acid fraction from heart of pregnant rats fed 50μg g^{-1} body weight clove or nutmeg volatile oils.

		Clove	*Nutmeg*
Fatty Acid	*Control*	*50μg g^{-1}*	*50μg g^{-1}*
12:0	tr.	tr.	tr.
14:0	0.46±0.03	0.38±0.02	0.42±0.04
16:0	15.7±0.37	14.5±0.62	15.6±0.56
17:0	0.38±0.02	0.41±0.05	0.45±0.03
18:0	17.3±0.88	18.3±0.53	20.3±0.76[a]
20:0	0.08±0.00	0.11±0.01	tr.
Total Saturates	***33.9***	***33.7***	***36.7***
14:1 *n-5*	tr.	tr.	tr.[c]
16:1 *n-7*	0.33±0.03	0.56±0.07[a]	0.86±0.03[c]
17:1 *n-7*	0.13±0.01	0.26±0.07	tr.[c]
18:1 *n-9*	9.33±0.38	10.3±0.99	14.9±1.60[b]
18:1 *n-7*	3.24±0.14	3.29±0.16	3.58±0.24
20:1 *n-9*	0.34±0.04	0.33±0.03	0.37±0.02
22:1 *n-9*	0.07±0.02	tr.	tr.
Total Monoenes	***13.4***	***14.7***	***19.7***
18:2 *n-6*	22.3±1.31	22.9±0.54	21.6±0.78
18:3 *n-6*	0.20±0.03	0.26±0.05	0.21±0.07
20:2 *n-6*	1.21±0.15	0.44±0.04[c]	1.16±0.10
20:3 *n-6*	0.47±0.03	0.43±0.03	0.34±0.04[a]
20:4 *n-6*	15.3±0.58	14.5±0.78	14.6±0.57
22:4 *n-6*	0.99±0.07	0.85±0.02	0.72±0.08[a]
Total n-6 PUFAs	***40.4***	***39.4***	***38.6***
18:3 *n-3*	tr.	tr.	0.49±0.03[c]
18:4 *n-3*	0.52±0.02	0.74±0.11	tr.[c]
20:5 *n-3*	0.27±0.03	0.26±0.02	tr.[c]
22:3 *n-3*	tr.	0.96±0.58[b]	tr.
22:5 *n-3*	2.00±0.12	1.88±0.15	1.46±0.20
22:6 *n-3*	9.75±0.36	8.66±0.16[a]	7.03±0.54[b]
Total n-3 PUFAs	***12.5***	***12.5***	***8.98***
Total PUFA	***53.0***	***51.9***	***47.6***
U/S ratio[1]	***0.40***	***0.44***	***0.54***
P/S ratio[2]	***1.56***	***1.54***	***1.30***
n-6/n-3 ratio[3]	***3.23***	***3.15***	***4.03***

The results are expressed as percentage composition and are means ± S.E.M. (n=5). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] P<0.05; [b] P<0.01; [c] P<0.001. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 41. Fatty acid composition (percentage of major acids by weight) of the phospholipid fraction from heart of pregnant rats fed 50μg g^{-1} body weight clove or nutmeg volatile oils.

		Clove	*Nutmeg*
Fatty Acid	*Control*	*50μg g^{-1}*	*50μg g^{-1}*
12:0	tr.	tr.	tr.
14:0	tr.	tr.	0.11±0.02[b]
16:0	14.2±0.58	13.3±0.60	13.3±0.36
17:0	0.39±0.02	0.41±0.01	0.38±0.01
18:0	24.9±0.05	25.3±1.16	27.9±0.37[c]
20:0	tr.	0.16±0.02[b]	0.21±0.03[c]
Total Saturates	***39.5***	***39.3***	***41.9***
14:1 *n-5*	tr.	tr.	tr.
16:1 *n-7*	0.26±0.04	0.38±0.04	0.29±0.03
17:1 *n-7*	tr.	0.17±0.02[b]	0.17±0.02[b]
18:1 *n-9*	2.64±0.04	3.31±0.23	3.23±0.11[c]
18:1 *n-7*	3.55±0.20	4.16±0.15	4.00±0.05[b]
20:1 *n-9*	tr.	0.18±0.02[b]	0.15±0.02[b]
22:1 *n-9*	tr.	tr.	tr.
Total Monoenes	***6.45***	***8.20***	***7.84***
18:2 *n-6*	15.6±0.56	19.5±1.31[a]	15.8±0.45
18:3 *n-6*	tr.	0.17±0.04[b]	0.21±0.03
20:2 *n-6*	0.23±0.02	0.26±0.03	0.25±0.01
20:3 *n-6*	0.27±0.02	0.39±0.02[a]	0.26±0.01
20:4 *n-6*	17.9±0.58	17.2±1.23	17.3±0.51
22:4 *n-6*	0.65±0.03	0.47±0.07	0.60±0.03
Total n-6 PUFAs	***34.6***	***37.9***	***34.4***
18:3 *n-3*	tr.	tr.	0.10±0.01[b]
18:4 *n-3*	tr.	0.16±0.00	0.08±0.01
20:5 *n-3*	0.13±0.04	0.18±0.01	0.16±0.02
22:3 *n-3*	tr.	tr.	tr.
22:5 *n-3*	1.73±0.11	2.46±0.23[a]	1.38±0.10[a]
22:6 *n-3*	16.2±0.59	20.6±1.38	13.1±0.30[c]
Total n-3 PUFAs	***18.0***	***23.4***	***14.8***
Total PUFA	***52.7***	***61.3***	***49.2***
U/S ratio[1]	***0.16***	***0.21***	***0.19***
P/S ratio[2]	***1.33***	***1.56***	***1.17***
n-6/n-3 ratio[3]	***1.92***	***1.62***	***2.32***

The results are expressed as percentage composition and are means ± S.E.M. (n=5). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 42. Fatty acid composition (percentage of major acids by weight) of the triacylglyceride fraction from kidney of pregnant rats fed 50μg g^{-1} body weight clove or nutmeg volatile oils.

		Clove	*Nutmeg*
Fatty Acid	*Control*	*50μg g^{-1}*	*50μg g^{-1}*
12:0	tr.	tr.	tr.
14:0	1.07±0.06	2.26±0.23[b]	0.99±0.13
16:0	25.8±0.60	26.6±0.98	26.3±1.33
17:0	0.23±0.02	0.21±0.03	0.25±0.02
18:0	4.69±0.22	10.9±1.80[c]	5.16±0.31
20:0	0.13±0.03	tr.[c]	0.12±0.01
Total Saturates	***31.9***	***40.0***	***32.8***
14:1 *n-5*	tr.	tr.	tr.
16:1 *n-7*	5.55±0.29	3.22±0.35[b]	4.21±0.40[a]
17:1 *n-7*	0.24±0.02	0.18±0.02	0.20±0.01
18:1 *n-9*	31.3±0.87	28.4±2.25	30.6±1.06
18:1 *n-7*	2.78±0.05	2.71±0.19	2.99±0.05[a]
20:1 *n-9*	0.53±0.06	0.37±0.13	0.19±0.02[c]
22:1 *n-9*	tr.	tr.	0.07±0.03
Total Monoenes	***40.4***	***34.8***	***38.3***
18:2 *n-6*	24.4±0.87	23.4±2.77	25.7±0.58
18:3 *n-6*	0.11±0.01	0.12±0.03	0.09±0.01[a]
20:2 *n-6*	0.19±0.00	tr.[c]	0.25±0.03
20:3 *n-6*	0.13±0.00	0.13±0.00	0.14±0.02
20:4 *n-6*	0.80±0.10	1.52±0.05[a]	0.66±0.08
22:4 *n-6*	tr.	tr.	0.15±0.02[2]
Total n-6 PUFAs	***25.7***	***34.2***	***27.0***
18:3 *n-3*	tr.	tr.	1.38±0.07[c]
18:4 *n-3*	1.69±0.07	1.66±0.09	tr.[c]
20:5 *n-3*	0.11±0.01	0.14±0.10	0.11±0.01
22:3 *n-3*	tr.	tr.	tr.
22:5 *n-3*	0.24±0.02	0.29±0.15	0.18±0.01
22:6 *n-3*	0.64±0.06	1.56±0.38[c]	0.60±0.05
Total n-3 PUFAs	***2.76***	***3.65***	***2.27***
Total PUFA	***28.4***	***37.9***	***29.3***
U/S ratio[1]	***1.27***	***0.87***	***1.17***
P/S ratio[2]	***0.89***	***0.95***	***0.89***
n-6/n-3 ratio[3]	***9.31***	***9.32***	***11.9***

The results are expressed as percentage composition and are means ± S.E.M. (n=5). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] P<0.05; [b] P<0.01; [c] P<0.001. tr.:<0.05% weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 43. Fatty acid composition (percentage of major acids by weight) of the free fatty acid fraction from kidney of pregnant rats fed 50μg g^{-1} body weight clove or nutmeg volatile oils.

		Clove	*Nutmeg*
Fatty Acid	*Control*	*50μg g^{-1}*	*50μg g^{-1}*
12:0	tr.	tr.	tr.
14:0	0.18±0.03	1.08±0.06[c]	0.21±0.09
16:0	16.2±0.61	24.3±0.33[c]	17.6±1.04
17:0	0.28±0.02	0.24±0.03	0.22±0.02
18:0	12.2±0.44	4.09±0.33[c]	11.9±1.72
20:0	0.12±0.00	0.09±0.01[a]	0.13±0.02
Total Saturates	***29.0***	***29.8***	***30.0***
14:1 *n-5*	tr.	tr.	tr.
16:1 *n-7*	0.40±0.12	4.76±0.74[c]	0.86±0.06[a]
17:1 *n-7*	0.14±0.02	0.25±0.01[a]	tr.[c]
18:1 *n-9*	15.4±0.51	27.8±1.39[c]	19.3±0.43[a]
18:1 *n-7*	2.59±0.06	2.75±0.16	2.89±0.11
20:1 *n-9*	0.42±0.02	0.55±0.09	0.43±0.01
22:1 *n-9*	0.07±0.00	tr.	tr.
Total Monoenes	***19.0***	***36.7***	***23.5***
18:2 *n-6*	19.3±0.48	29.0±1.38[c]	23.4±1.38[b]
18:3 *n-6*	0.14±0.01	0.18±0.01	tr.
20:2 *n-6*	1.16±0.07	tr.[c]	0.95±0.06
20:3 *n-6*	1.02±0.04	0.18±0.02[c]	0.84±0.10
20:4 *n-6*	20.6±0.40	0.81±0.15[c]	17.9±0.35[b]
22:4 *n-6*	0.74±0.03	tr.[c]	0.60±0.09
Total n-6 PUFAs	***42.9***	***30.2***	***43.7***
18:3 *n-3*	tr.	tr.	1.18±0.05[c]
18:4 *n-3*	0.72±0.06	2.05±0.05[c]	tr.[c]
20:5 *n-3*	1.20±0.09	0.18±0.04[c]	1.08±0.11
22:3 *n-3*	tr.	0.16±0.00[c]	tr.
22:5 *n-3*	0.60±0.17	0.42±0.07	1.23±0.23[b]
22:6 *n-3*	5.96±0.31	0.95±0.23[c]	4.72±0.06[a]
Total n-3 PUFAs	***8.48***	***3.76***	***8.21***
Total PUFA	***51.4***	***33.9***	***51.9***
U/S ratio[1]	***0.66***	***1.23***	***0.78***
P/S ratio[2]	***1.77***	***1.14***	***1.73***
n-6/n-3 ratio[3]	***5.06***	***8.03***	***5.32***

The results are expressed as percentage composition and are means ± S.E.M. (n=5). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05% weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 44. Fatty acid composition (percentage of major acids by weight) of the phospholipid fraction from kidney of pregnant rats fed 50μg g^{-1} body weight clove or nutmeg volatile oils.

		Clove	*Nutmeg*
Fatty Acid	*Control*	*50μg g^{-1}*	*50μg g^{-1}*
12:0	tr.	tr.	tr.
14:0	0.15±0.03	0.18±0.09	0.14±0.01
16:0	21.4±0.84	20.0±0.73	23.1±0.58
17:0	0.34±0.02	0.32±0.04	0.31±0.02
18:0	18.3±0.17	19.1±0.42	20.2±0.73[b]
20:0	0.20±0.01	0.10±0.06[a]	0.17±0.01
Total Saturates	***40.4***	***39.8***	***43.9***
14:1 *n-5*	tr.	tr.	tr.
16:1 *n-7*	0.26±0.05	0.59±0.08[a]	0.73±0.08[c]
17:1 *n-7*	0.19±0.00	tr.[c]	0.17±0.02
18:1 *n-9*	7.52±0.23	8.74±0.73	8.81±0.27[b]
18:1 *n-7*	2.51±0.09	2.79±0.09	3.03±0.05[b]
20:1 *n-9*	0.16±0.02	0.21±0.00	0.20±0.02
22:1 *n-9*	tr.	tr.	tr.
Total Monoenes	***10.6***	***12.3***	***12.9***
18:2 *n-6*	12.5±0.46	16.2±0.34[a]	14.4±0.27[b]
18:3 *n-6*	tr.	tr.	tr.
20:2 *n-6*	0.30±0.02	0.32±0.00	0.33±0.02
20:3 *n-6*	0.67±0.04	0.66±0.09	0.57±0.02
20:4 *n-6*	26.1±1.43	24.8±0.74	22.6±0.82
22:4 *n-6*	0.37±0.01	tr.[c]	0.35±0.02
Total n-6 PUFAs	***39.9***	***42.0***	***38.3***
18:3 *n-3*	tr.	tr.	0.23±0.02[c]
18:4 *n-3*	0.21±0.02	0.35±0.07	tr.[c]
20:5 *n-3*	1.28±0.05	1.21±0.12	0.85±0.02[c]
22:3 *n-3*	tr.	tr.	tr.
22:5 *n-3*	0.57±0.03	0.55±0.03	0.41±0.02[b]
22:6 *n-3*	6.33±0.39	5.60±0.30	3.99±0.20[c]
Total n-3 PUFAs	***8.39***	***7.71***	***5.48***
Total PUFA	***48.4***	***49.7***	***43.7***
U/S ratio[1]	***0.26***	***0.31***	***0.29***
P/S ratio[2]	***1.20***	***1.25***	***1.00***
n-6/n-3 ratio[3]	***4.76***	***5.45***	***6.99***

The results are expressed as percentage composition and are means ± S.E.M. (n=5). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 45. Fatty acid composition (percentage of major acids by weight) of the cholesteryl ester fraction from liver of pregnant rats fed 50μg g^{-1} body weight clove or nutmeg volatile oils.

		Clove	*Nutmeg*
Fatty Acid	*Control*	*50μg g^{-1}*	*50μg g^{-1}*
12:0	tr.	tr.	tr.
14:0	tr.	tr.	tr.
16:0	33.5±2.10	27.6±2.17	27.4±3.07
17:0	tr.	0.50±0.07[c]	0.39±0.05[c]
18:0	10.5±1.11	10.6±0.30	9.85±1.44
20:0	tr.	tr.	tr.
Total Saturates	***44.0***	***38.7***	***37.6***
14:1 *n-5*	tr.	tr.	tr.
16:1 *n-7*	2.34±0.01	1.62±0.52[a]	1.06±0.14[b]
17:1 *n-7*	tr.	0.25±0.05[b]	tr.
18:1 *n-9*	12.5±0.13	15.0±1.34	15.0±1.39
18:1 *n-7*	1.44±0.11	1.93±0.15[a]	1.52±0.06
20:1 *n-9*	tr.	0.39±0.14[b]	0.50±0.09[b]
22:1 *n-9*	tr.	tr.	tr.
Total Monoenes	***16.3***	***19.2***	***18.1***
18:2 *n-6*	23.1±2.00	23.1±1.23	22.3±1.27
18:3 *n-6*	tr.	tr.	0.33±0.02[b]
20:2 *n-6*	0.70±0.19	0.33±0.03[a]	0.68±0.07
20:3 *n-6*	tr.	0.27±0.04[b]	tr.
20:4 *n-6*	10.3±1.00	9.60±0.37	14.0±3.22
22:4 *n-6*	tr.	tr.	tr.
Total n-6 PUFAs	***34.2***	***33.3***	***37.4***
18:3 *n-3*	tr.	tr.	1.11±0.12[c]
18:4 *n-3*	1.51±0.03	1.28±0.28	tr.[c]
20:5 *n-3*	tr.	0.77±0.13[c]	0.98±0.70[c]
22:3 *n-3*	tr.	tr.	tr.
22:5 *n-3*	1.56±0.28	1.21±0.09	2.02±0.51
22:6 *n-3*	3.69±0.89	5.42±0.91[a]	6.55±1.97[b]
Total n-3 PUFAs	***6.76***	***8.68***	***10.7***
Total PUFA	***40.9***	***41.9***	***48.0***
U/S ratio[1]	***0.37***	***0.50***	***0.48***
P/S ratio[2]	***0.93***	***1.08***	***1.28***
n-6/n-3 ratio[3]	***5.06***	***3.84***	***3.50***

The results are expressed as percentage composition and are means ± S.E.M. (n=5). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] P<0.05; [b] P<0.01; [c] P<0.001. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 46. Fatty acid composition (percentage of major acids by weight) of the triacylglyceride fraction from liver of pregnant rats fed 50μg g^{-1} body weight clove or nutmeg volatile oils.

		Clove	*Nutmeg*
Fatty Acid	*Control*	*50μg g^{-1}*	*50μg g^{-1}*
12:0	tr.	tr.	tr.
14:0	0.61±0.14	0.36±0.02	0.28±0.05
16:0	28.7±1.17	30.8±0.41	27.8±4.01
17:0	0.30±0.03	0.40±0.09	0.21±0.01
18:0	3.31±0.28	7.88±1.10[c]	3.19±0.89
20:0	tr.	tr.	tr.
Total Saturates	***32.9***	***39.4***	***31.5***
14:1 *n-5*	tr.	tr.	tr.
16:1 *n-7*	2.37±0.44	1.86±0.40	1.03±0.18[a]
17:1 *n-7*	0.17±0.03	0.15±0.05	tr.[c]
18:1 *n-9*	21.7±1.23	21.9±0.54	10.6±0.50[c]
18:1 *n-7*	2.28±0.12	2.46±0.43	1.86±0.25
20:1 *n-9*	0.38±0.07	0.29±0.06	0.20±0.10
22:1 *n-9*	tr.	tr.	tr.
Total Monoenes	***26.9***	***26.7***	***13.7***
18:2 *n-6*	29.3±1.06	24.7±1.14[a]	32.7±2.28
18:3 *n-6*	0.36±0.08	0.31±0.04	0.28±0.10
20:2 *n-6*	0.28±0.02	0.41±0.01[b]	0.21±0.03
20:3 *n-6*	0.30±0.08	0.60±0.08[c]	0.33±0.09
20:4 *n-6*	2.94±0.88	tr.[c]	3.12±0.09
22:4 *n-6*	0.48±0.28	0.46±0.08	0.39±0.11
Total n-6 PUFAs	***33.7***	***26.5***	***37.0***
18:3 *n-3*	0.09±0.02	tr.[c]	1.68±0.15[c]
18:4 *n-3*	1.65±0.27	1.27±0.26	tr.[c]
20:5 *n-3*	0.60±0.22	0.76±0.11	0.96±0.12
22:3 *n-3*	tr.	tr.	tr.
22:5 *n-3*	1.93±0.20	0.86±0.13[b]	1.47±0.14
22:6 *n-3*	5.49±1.08	3.26±0.77	8.98±0.22
Total n-3 PUFAs	***9.76***	***6.15***	***13.09***
Total PUFA	***43.4***	***32.6***	***50.1***
U/S ratio[1]	***0.82***	***0.68***	***0.44***
P/S ratio[2]	***1.32***	***0.83***	***1.59***
n-6/n-3 ratio[3]	***3.45***	***4.31***	***2.83***

The results are expressed as percentage composition and are means ± S.E.M. (n=5). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 47. Fatty acid composition (percentage of major acids by weight) of the free fatty acid fraction from liver of pregnant rats fed 50μg g^{-1} body weight clove or nutmeg volatile oils.

		Clove	*Nutmeg*
Fatty Acid	*Control*	*50μg g^{-1}*	*50μg g^{-1}*
12:0	tr.	tr.	tr.
14:0	0.42±0.08	0.22±0.04[a]	0.32±0.04
16:0	24.9±2.10	21.6±1.89	23.5±0.72
17:0	0.35±0.03	0.40±0.04	0.26±0.02[a]
18:0	6.43±0.55	14.51±0.93[c]	8.23±1.38
20:0	tr.	tr.	tr.
Total Saturates	***32.1***	***36.7***	***32.4***
14:1 *n-5*	tr.	tr.	tr.
16:1 *n-7*	0.95±0.16	1.41±0.17[a]	0.70±0.15
17:1 *n-7*	tr.	0.13±0.03[c]	0.12±0.02[c]
18:1 *n-9*	14.1±1.46	13.7±0.35	10.7±0.78[a]
18:1 *n-7*	1.96±0.12	2.04±0.29	1.56±0.09[a]
20:1 *n-9*	tr.	0.26±0.03[c]	0.23±0.02[c]
22:1 *n-9*	tr.	tr.	tr.
Total Monoenes	***16.9***	***17.5***	***13.4***
18:2 *n-6*	27.3±.01	20.2±0.81[a]	23.2±1.51
18:3 *n-6*	0.49±0.07	0.47±0.09	0.22±0.04[b]
20:2 *n-6*	0.54±0.08	0.33±0.03	0.32±0.03[a]
20:3 *n-6*	0.69±0.08	0.41±0.05	0.36±0.05[b]
20:4 *n-6*	7.09±0.34	11.1±0.88[a]	10.2±0.63[b]
22:4 *n-6*	0.50±0.07	0.72±0.21	0.85±0.23
Total n-6 PUFAs	***36.6***	***33.2***	***35.2***
18:3 *n-3*	tr.	tr.	1.16±0.24[c]
18:4 *n-3*	1.77±0.36	1.23±0.05	tr.[c]
20:5 *n-3*	0.50±0.06	0.66±0.15	0.61±0.11
22:3 *n-3*	tr.	0.23±0.08[c]	tr.
22:5 *n-3*	2.13±0.38	1.99±0.23	2.14±0.30
22:6 *n-3*	9.33±0.05	11.9±0.38[a]	12.8±0.91[a]
Total n-3 PUFAs	***13.7***	***16.0***	***16.7***
Total PUFA	***50.3***	***49.3***	***51.9***
U/S ratio[1]	***0.53***	***0.48***	***0.41***
P/S ratio[2]	***1.57***	***1.34***	***1.60***
n-6/n-3 ratio[3]	***2.67***	***2.08***	***2.11***

The results are expressed as percentage composition and are means ± S.E.M. (n=5). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] P<0.05; [b] P<0.01; [c] P<0.001. tr.:<0.05%weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Appendix 48. Fatty Acid Composition (Percentage of Major Acids by Weight) of the Phospholipid Fraction from Liver of Pregnant Rats Fed 50μg g^{-1} Body Weight Clove or Nutmeg Volatile Oils.

		Clove	*Nutmeg*
Fatty Acid	*Control*	*50μg g^{-1}*	*50μg g^{-1}*
12:0	tr.	tr.	tr.
14:0	0.07±0.01	tr.[b]	tr.[b]
16:0	19.8±1.06	19.3±1.31	22.1±1.26
17:0	0.39±0.03	0.39±0.06	0.32±0.20
18:0	24.2±0.76	24.9±0.47	26.6±1.08
20:0	tr.	tr.	tr.
Total Saturates	***44.4***	***44.6***	***49.0***
14:1 *n-5*	tr.	tr.	tr.
16:1 *n-7*	0.33±0.05	0.27±0.01	0.30±0.05
17:1 *n-7*	tr.	0.13±0.01[b]	0.11±0.08[b]
18:1 *n-9*	2.48±0.22	2.78±0.29	2.06±0.43
18:1 *n-7*	1.41±0.14	1.49±0.14	1.65±0.29
20:1 *n-9*	0.07±0.01	tr.[b]	tr.[b]
22:1 *n-9*	tr.	tr.	tr.
Total Monoenes	***4.29***	***4.67***	***4.12***
18:2 *n-6*	11.4±0.91	11.3±1.14	7.7±0.36[b]
18:3 *n-6*	0.09±0.00	0.17±0.04[b]	0.17±0.14
20:2 *n-6*	0.31±0.03	0.34±0.02	0.31±0.05
20:3 *n-6*	0.81±0.14	0.27±0.09[b]	0.34±0.00
20:4 *n-6*	15.7±0.52	15.9±0.94	15.4±1.09
22:4 *n-6*	0.32±0.03	0.28±0.01	0.31±0.02
Total n-6 PUFAs	***28.6***	***28.3***	***24.2***
18:3 *n-3*	tr.	tr.	tr.
18:4 *n-3*	0.14±0.01	0.16±0.02	tr.[c]
20:5 *n-3*	0.36±0.07	0.34±0.06	0.13±0.09
22:3 *n-3*	tr.	tr.	tr.
22:5 *n-3*	1.03±0.05	1.19±0.09	0.67±0.24[b]
22:6 *n-3*	21.4±1.20	22.1±1.69	21.5±1.48
Total n-3 PUFAs	***22.9***	***23.8***	***22.3***
Total PUFA	***51.5***	***52.0***	***46.5***
U/S ratio[1]	***0.10***	***0.11***	***0.08***
P/S ratio[2]	***1.16***	***1.17***	***0.95***
n-6/n-3 ratio[3]	***1.25***	***1.19***	***1.09***

The results are expressed as percentage composition and are means ± S.E.M. (n=5). Significance of differences due to the diet were determined by the Student's *t*-test using a pooled standard deviation, indicated as [a] P<0.05; [b] P<0.01; [c] P<0.001. tr.:<0.05% weight. PUFAs: polyunsaturated fatty acids. [1]: unsaturate: saturate ratio; [2]: polyunsaturate: saturate ratio; [3]:*n-6* PUFA: *n-3* PUFA ratio.

Publications

Abstracts

1. **Dorman, H.J.D., Youdim, K.A., Deans, S.G., and Lis-Balchin, M.** [1995]. Antioxidant-rich plant volatile oils: *In vitro* assessment of activity. *Proceedings of the 26th International Symposium on Essential Oils, Hamburg, Germany*, P40.

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Reviews

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