

Essential Oils in Food Processing

Chemistry, Safety and Applications

Edited by

Seyed Mohammad Bagher Hashemi, Amin Mousavi Khaneghah
and Anderson de Souza Sant'Ana

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Essential Oils in Food Processing



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Essential Oils in Food Processing

Chemistry, Safety and Applications

Edited by

Seyed Mohammad Bagher Hashemi

Fasa University

Iran

Amin Mousavi Khaneghah

University of Campinas

Brazil

Anderson de Souza Sant'Ana

University of Campinas

Brazil

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This edition first published 2018
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Editorial Office

9600 Garsington Road, Oxford, OX4 2DQ, UK

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Library of Congress Cataloging-in-Publication Data

Names: Hashemi, Seyed Mohammad Bagher, editor. | Khaneghah, Amin Mousavi, editor. | Sant'Ana, Anderson de Souza, editor.

Title: Essential oils in food processing : chemistry, safety and applications / edited by Seyed Mohammad Bagher Hashemi, Amin Mousavi Khaneghah, Anderson de Souza Sant'Ana.

Description: Hoboken, NJ : John Wiley & Sons, 2018. | Series: IFT Press Series |

Includes bibliographical references and index. |

Identifiers: LCCN 2017030692 (print) | LCCN 2017043887 (ebook) | ISBN 9781119149354 (pdf) |

ISBN 9781119149378 (epub) | ISBN 9781119149347 (cloth)

Subjects: LCSH: Essences and essential oils—Industrial applications. | Food industry and trade. |

Food additives industry.

Classification: LCC TP958 (ebook) | LCC TP958 .E875 2018 (print) | DDC 664/.06—dc23

LC record available at <https://lccn.loc.gov/2017030692>

Cover Design: Wiley

Cover Image: © botamochi/iStockphoto

Set in 10/12pt Warnock by SPi Global, Pondicherry, India

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List of Contributors

Hamid Akbariirad

Department of Food Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran

Saeedeh Shojaee-Aliabadi

Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Riccardo Amorati

Department of Chemistry 'G. Ciamician', University of Bologna, Italy

Francisco J. Barba

Universitat de València, Faculty of Pharmacy, Nutrition and Food Science Area, Valencia, Spain

Antonio Bevilacqua

Department of the Science of Agriculture, Food and Environment (SAFE), University of Foggia, Italy

Farid Chemat

Université d'Avignon et des Pays de Vaucluse, France

Maria Rosaria Corbo

Department of the Science of Agriculture, Food and Environment (SAFE), University of Foggia, Italy

Duarte, M.C.T.

Research Centre for Chemistry, Biology and Agriculture – CPQBA/UNICAMP – Microbiology Division, Campinas, São Paulo, Brazil

Duarte, R.M.T.

Research Centre for Chemistry, Biology and Agriculture – CPQBA/UNICAMP – Microbiology Division, Campinas, São Paulo, Brazil

Ismail Es

Department of Material and Bioprocess Engineering, Faculty of Chemical Engineering, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil

Hassan Eslahi

Department of Chemistry, College of Sciences, Shiraz University, Iran

Nafiseh Fahimi

Department of Chemistry, College of Sciences, Shiraz University, Iran

Mario C. Foti

Istituto di Chimica Biomolecolare del CNR, Italy

Ralf Greiner

Department of Food Technology and Bioprocess Engineering, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Germany

Seyed Mohammad Bagher Hashemi

Department of Food Science and Technology, College of Agriculture, Fasa University, Fasa, Iran

Seyede Marzieh Hosseini

Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Amin Mousavi Khaneghah

Department of Food Science, Faculty of Food Engineering, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil

Anissa Khelfa

Sorbonne Universités, Université de Technologie de Compiègne, Laboratoire Transformations Intégrées de la Matière Renouvelable, France

Shima Bazgir Khorram

Department of Food Science and Technology, College of Agriculture, Fasa University, Fasa, Iran

Mohamed Koubaa

Sorbonne Universités, Université de Technologie de Compiègne, Laboratoire Transformations Intégrées de la Matière Renouvelable, France

Ramadasan Kuttan

Department of Biochemistry, Amala Cancer Research Centre, Kerala, India

Sze Ying Leong

Department of Food Science, University of Otago, New Zealand;
Department of Safety and Quality of Fruit and Vegetables, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Germany

Vijayasteltar B. Liju

Department of Biochemistry, Amala Cancer Research Centre, Kerala, India

Aurelio López-Malo

Departamento de Ingeniería Química y Alimentos, Universidad de las Américas Puebla, Puebla, Mexico

Ana Cecilia Lorenzo-Leal

Departamento de Ingeniería Química y Alimentos, Universidad de las Américas Puebla, Puebla, Mexico

Emma Mani-López

Departamento de Ingeniería Química y Alimentos, Universidad de las Américas Puebla, Puebla, Mexico

Jairo Rene Martinez

Research Centre for Biomolecules, CIBIMOL-CENIVAM, Universidad Industrial de Santander, Bucaramanga, Colombia

Liela Mirmoghtadaie

Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Enrique Palou

Departamento de Ingeniería Química y Alimentos, Universidad de las Américas Puebla, Puebla, Mexico

Marianne Perricone

Department of the Science of Agriculture, Food and Environment (SAFE), University of Foggia, Italy

Rodrigues, M.V.N.

Research Centre for Chemistry, Biology and Agriculture – CPQBA/UNICAMP – Chemistry of Natural Products Division, Campinas, São Paulo, Brazil

Rodrigues, R.A.F.

Research Centre for Chemistry, Biology and Agriculture – CPQBA/UNICAMP – Organic Chemistry and Pharmaceutical Division, Campinas, São Paulo, Brazil

Shahin Roohinejad

Department of Food Technology and Bioprocess Engineering, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Germany;
Burn and Wound Healing Research Centre, Division of Food and Nutrition, Shiraz University of Medical Sciences, Iran

Anderson de Souza Sant'Ana

Department of Food Science, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil

Ali Reza Sardarian

Department of Chemistry, College of Sciences, Shiraz University, Iran

Milena Sinigaglia

Department of the Science of Agriculture, Food and Environment (SAFE), University of Foggia, Italy

Maryam Sohrabi

Department of Food Science and Technology, College of Agriculture, Fasa University, Fasa, Iran

Barbara Speranza

Department of the Science of Agriculture, Food and Environment (SAFE), University of Foggia, Italy

Elena E. Stashenko

Research Centre for Biomolecules, CIBIMOL-CENIVAM, Universidad Industrial de Santander, Bucaramanga, Colombia

Iuliana Vintilă

Food Science, Food Engineering and Applied Biotechnology Department, University 'Dunărea de Jos' Galați, România

Acknowledgements

Seyed Mohammad Bagher Hashemi would like to thank Allah for the opportunity to gain knowledge to write this book. He sincerely acknowledges the sacrifices made by his parents during his education. He also would like to express his appreciation to Fasa University.

Amin Mousavi Khaneghah and Anderson de Souza Sant'Ana gratefully acknowledge University of Campinas (UNICAMP), Campinas, São Paulo, Brazil.

Amin Mousavi Khaneghah likes to thank the support of CNPq-TWAS Postgraduate Fellowship (Grant # 3240274290).

Anderson de Souza Sant'Ana would like to thank the support of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant #CNPq 302763/2014-7).

Introduction

Amin Mousavi Khaneghah, Anderson de Souza Sant'Ana

In recent years, due to their application in therapeutic, food, cosmetic, aromatic, fragrance and spiritual uses, the essential oil industry has gained increased importance. Essential oils are complex mixtures of small molecular weight compounds including saturated and unsaturated hydrocarbons, alcohol, aldehydes, esters, ethers, ketones, oxides phenols and terpenes. Because of their natural origin and of their widely described potential beneficial properties, essential oils can find several applications in the field of food processing.

The use of natural compounds for food applications has attracted more attention in the last years due to the perception of people about synthetic food additives. Among the beneficial properties of essential oils, their antimicrobial activity can be considered one of the main factors driving their application in foods. The antimicrobial property of an essential oil is known to be influenced by composition, configuration, amount and their possible interaction. Despite the antimicrobial properties of some essential oils, to achieve the desired results regarding antimicrobial effectiveness, high concentrations are required that ending up limiting their extensive application in food formulations. Despite this, essential oils can serve as additional preservative factors using the concept of hurdle technology. In addition to the antimicrobial properties, the antioxidant properties of essential oils can be considered another high relevant beneficial property of these natural products. As synthetic antioxidants (*i.e.*, BHA) are recently suspected to be potentially harmful to human health, essential oils with their antioxidant activity are found to be a better choice to reduce oxidative reactions in foods. For their application in foods and other matrixes, it is essential to ensure the properties are not lost from obtainment to food formulation, processing and storage. In this context, extraction processes play a major role to ensure essential oils have the expected results when added to foods. Extraction processes are the most time- and effort-consuming processes for obtainment of essential oils. There are several methods to extract oils from their natural sources, such as distillation and expression, are considered as two key methods for extraction of the essential oils. The extraction method has been reported to affect the essential oil composition significantly. Moreover, there are other factors that also influence the chemistry of essential oils including plant variety, growth conditions (*e.g.*, fertilisers, climate), storage and so on. Therefore, to ensure a stable composition and the resulting properties of essential oils remains a complicated issue.

This book provides a comprehensive perspective of the pertinent aspects of essential oils including antimicrobial, toxicology, chemistry, extraction methods, composition, applications and other practical topics required for the development of this industry and wide application of essential oils.

Essential Oils and Their Characteristics

M.C.T. Duarte¹, R.M.T. Duarte¹, R.A.F. Rodrigues² and M.V.N. Rodrigues³

¹Research Centre for Chemistry, Biology and Agriculture – CPQBA/UNICAMP – Microbiology Division, Campinas, São Paulo, Brazil

²Research Centre for Chemistry, Biology and Agriculture – CPQBA/UNICAMP – Chemistry of Natural Products Division, Campinas, São Paulo, Brazil

³Research Centre for Chemistry, Biology and Agriculture – CPQBA/UNICAMP – Organic Chemistry and Pharmaceutical Division, Campinas, São Paulo, Brazil

1.1 Introduction

Essential oils are the main raw material for the aroma and fragrance, food and pharmaceutical industries. They have important biological activities that have been disclosed often in recent years. However, as the industry seeks its practical application and the development of new natural drugs containing active compounds from essential oils, there is an urgent need to standardise the plant material source. For this to become achievable, it is necessary to know the different factors that affect the production of essential oils by plants, in terms of its quantity as well as its quality.

It is known that plants produce essential oils as secondary metabolites in response to a physiological stress, pathogen attack and ecological factors. Also, in nature, the essential oils are recognised as defence compounds and attractors of pollinators, facilitating the reproduction of the vegetal species. The environmental variations, in turn, are also important in a plant's ability to produce these compounds. Considering all of these factors, the main problems related to the cultivation of aromatic plants are due to variations that occur in quantitative and qualitative changes in the essential oils production. The main factors involved in the biosynthesis of essential oils by medicinal and aromatic plants are discussed in this chapter.

In order to optimise its commercial exploitation, the different factors involved in the production of essential oils must be taken into account, since the induction into its substance synthesis could affect the specific compounds of interest and their economic applications, as well as affecting the standard amount of produced oil.

1.1.1 Chemical Characteristics of Essential Oils

The designation *essential oil* originated from Aristotle's era, because the of the idea of life-essential elements — fire, air, earth and water. In this case, the fifth element was

considered to be the soul or the spirit of life. Distillation and evaporation were the processes of removing the soul from the plant or essential oils. Nowadays, these oils are also known as volatile oils, but far from being soul, essential oils are a complexity of aroma's composition. Those constituents of essential oils are generally derived from phenylpropanoid routes (Thayumanavan & Sadasivam, 2003).

The studies of those routes have disclosed the relevance of the aspect of physiology regulation, but certainly the isoprenoid exemplifies the major group of secondary metabolites in herbs, which exhibit extremely vast varieties of chemical structures and biochemical functions. Since primary metabolites exist in all plant cells that are qualified by division, secondary metabolites are there exclusive by accident, and are not essential for that herb. In contrariety to primary metabolites, secondary compounds vary extensively in their occurrence in those herbs and some may appear only in a unique or a few species (Krings & Berger, 1998).

Due to the connection of terpenoids in many pharmacological properties and their great value added specially for pharmaceutical, cosmetic and food industries, the isoprenoid route has been a spotlight for most related articles. Essential oils are nearly always rotational and have a high refractory index; they are sparingly soluble in water, usually less dense than water and liquid at room temperature, but there is some exception, as trans-anethole (anise camphor) from the oil of anise (*Pimpinella anisum L.*), and they may be classified using different criteria: consistency, origin and chemical nature. As stated by their consistency, essential oils are classified as essences, balsams or resins. Depending on their origin, essential oils are natural, artificial or synthetic. Essential oils are aromatic chemical compounds that came from plant's glands. Due to their volatility, flavour and toxicity, this class of compounds also plays significant aspects in the defence's herbs, communication between plants and pollinator attractiveness (Muñoz-Bertomeu *et al.*, 2007; Thayumanavan & Sadasivam, 2003).

A lot of herbs can be view as being composed of a basic unit called isoprene or isopentane. Terms such as isoprenoid or terpenoid are employed concurrently. Many terpenoids are assemble of carbon atoms from acyclic disposition to a cyclic disposition by different chemical reactions, like, condensation, addition, cyclisation, deletion or rearrangements to be transformed in a basic unit, and generally, are very extensively diffused throughout the total plant kingdom. These compounds comprise a structurally varied class that can be splitted into the main and the minor terpenoids (Daviet & Schalk, 2010; Muñoz-Bertomeu *et al.*, 2006; Daniel, 2006; Thayumanavan & Sadasivam, 2003).

Biosynthesis of terpenes can occur in distinct sector of the herb, such as bark, flowers, fruits, leaves, roots, ryzomes, seeds and wood, and have all been described to concentrate them in different herbs. Terpenes that are main metabolites include carotenes, regulators of growth, proteins, quinones, polyprenols and the sterol, substitutes of terpenes with an alcohol functional group (Daviet & Schalk, 2010). These constituents are indispensable for preserving the membrane to keep the entirety of its structure, also to protection against light, and securing the maintenance of its biological functionality. Terpenes are a large class of chemical compounds, classified by the molecular weight, being monoterpenes, sesquiterpenes, diterpenes, seterterpenes, triterpenes, tetraterpenes and phytosterols amongst others (Thayumanavan & Sadasivam, 2003).

Monoterpenes are the major contributor of most important essential oils in nature. Since the monoterpenes ($C_{10}H_{16}$) are small molecules with two isoprene units, such as menthol and linalool, and they are lipophilic; they are promptly consumed through the

skin. Synthetic compounds can be used to break down the problems come across with herbal products by creating actions for the construction of such molecules, regardless of the original species. Indeed, ways have been developed for most of the natural molecules, but, given their commonly complex spatial arrangements, the industrial production is not practicable for the majority of examples (Daviet & Schalk, 2010; Muñoz-Bertomeu *et al.*, 2006; Daniel, 2006; Thayumanavan & Sadasivam, 2003).

Characteristically, plant's secondary metabolites are cumulated and stored in relatively huge quantities, which can be explained by their role as chemical signals or defence compounds. Terpenes are built up from the union of the two carbon units with five members each by condensation, isopentenyl diphosphate synonym isopentenyl pyrophosphate (IPP) and dimethylallyldiphosphate synonym dimethylallyl pyrophosphate (DMAPP), with different modes of structure formation, number of unsaturated bonds and type of linker groups. Not all terpenoids have a composition of their structures in the repetition of five carbon atoms, as can be habitual of them, considering that they are formed usually from isoprene as forming matrix (Daniel, 2006; Thayumanavan & Sadasivam, 2003).

Terpenes consisting of more than five isoprene structures appear in all herbs, and simpler terpenes (C_{10} – C_{25}) are mainly restricted in the phylogeny classification to the vascular plants/higher plants or, synonym Tracheophyta, while sesquiterpenes have been found broadly in division Bryophyta and in the kingdom Fungi. Monoterpenoids are colourless, distilled by steam, liquids insoluble in water with a typical scent, with a range of boiling points of 140 until 180 °C. Some of them have shown potentiality as insect plague management because they simply provide herbs with defences against insects that feed from it. Many terpenes also operate as insect captivate, being green and innocuous to humans and other animals (Daniel, 2006; Thayumanavan & Sadasivam, 2003).

More than a thousand sesquiterpenes are known today. Sesquiterpenes are the biggest category of terpenoids with a broad molecular structures and are constitutes of three isoprene matrixes, that is, they are composed with 15 carbon atoms, such as farnesol, guaiazulene, bisabolol and become from medicinal plants in distilleries equipments, in the bitter-tasting substances and essential oils in a lot of herbs. Diterpenes are formed by 20 carbon atoms, by their condensation of four isoprene residues, such as taxol, gibberellins, phytol and fusicocsin. Like sesquiterpenes, we know a thousand or more C_{20} compounds in this category, which fit into 20 main typical skeletons. Triterpenes are categorised based on the linear composition or number of cyclic compounds actual. Triterpenes relate to an inharmonious compilation of chemical compounds, which are consider to be aquired from squalene; the C_{30} non cyclic component by types of rings and ligands. Pentacyclic triterpenes are usually distributed in vascular plants, occurring as glycosides with sugar ligands (saponins) or without sugar ligands (aglycones) (Daniel, 2006; Thayumanavan & Sadasivam, 2003).

Two routes have been extensively studied, leading to these precursors, through the mevalonate (typically known as MVA route; C_6) and the 1-deoxyxylulose-D-5-phosphate (coded as the DXP route) pathways. The DXP compound, also known as the 2-C-methyl-D-erythritol-4-phosphate or methylerythritol phosphate (coded as the MEP route), takes place in plant plastids (chloroplast) and also by bacteria, while the MVA track different sources such as fungus, in the herb's cytosol and in some animals. In the mevalonic acid route, the key enzyme is 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA

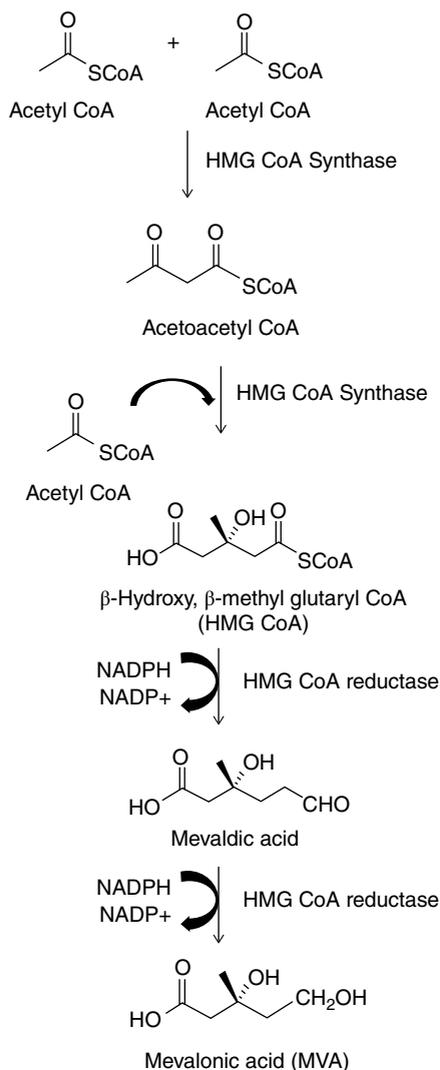


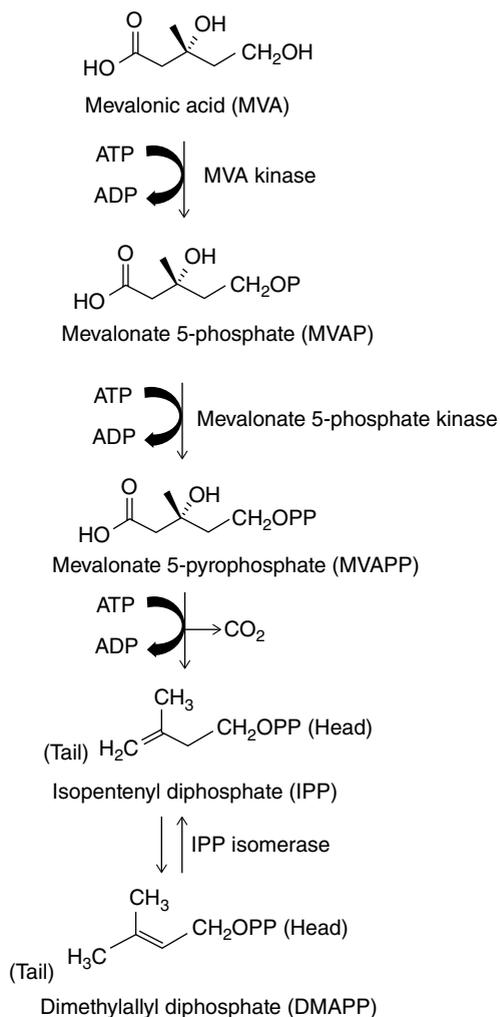
Figure 1.1 Synthesis of mevalonic acid in plants, from classical MVA pathway. HMG-CoA reductase: 3-hydroxy-3-methylglutaryl CoA reductase and HMG-CoA synthase: 3-hydroxy-3-methylglutaryl CoA synthase. From Thayumanavan & Sadasivam (2003).

reductase coded as HMGR). HMGR assemble the initial sequence of the MVA route by converting HMG-CoA to MVA using NADPH as coenzyme (Muñoz-Bertomeu *et al.*, 2007; Muñoz-Bertomeu *et al.*, 2006; Thayumanavan & Sadasivam, 2003).

The synthesis of mevalonic acid is illustrated in Figure 1.1.

In higher plants, the condensation of the basic C₅ units, isopentenyl diphosphate (coded as IPP) and dimethylallyldiphosphate (coded as DMAPP), is catalysed by the enzyme prenyltransferases (Figure 1.2), which builds the chain of prenyldiphosphate, designated as the original source for each category of terpenoids: geranyl diphosphate (coded as GPP; C₁₀), farnesyl diphosphate (coded as FPP; C₁₅) and geranylgeranyl diphosphate (coded as GGPP), respectively for the monoterpenes, sesquiterpenes and diterpenes (Figure 1.3) (Muñoz-Bertomeu *et al.*, 2007; Muñoz-Bertomeu *et al.*, 2006; Thayumanavan & Sadasivam, 2003).

Figure 1.2 Synthesis of isopentenyl diphosphate (IPP) and dimethylallyldiphosphate (DMAPP). From Thayumanavan & Sadasivam (2003).



The production of IPP and DMAPP proceeds via two alternative routes (Figure 1.4): the usual cytosolic mevalonate (MVA) route and the methylerythritol phosphate (MEP) route. The MEP route, confined in the plastids, is responsible to provide isopentenyl diphosphate and dimethylallyldiphosphate for monoterpene and sesquiterpene synthesis. The following achievement implicates the terpenoid synthases, which are presenting a vast enzyme group. Terpenoid synthases play an important function in the terpenoid synthesis, since they are responsible for the formation of farnesyl diphosphate, geranyl diphosphate and geranylgeranyl diphosphate to form the main structures of the terpenoids, and are thus at the source of the highly large number of possibilities of compounds such as squalene, generated by squalene synthase that catalyses the head-to-head reaction of two farnesyl diphosphate units, the initial important reaction in sterol category synthesis; end-product sterols (β -sitosterol and stigmasterol). Given the vast range of terpenoid configuration, many

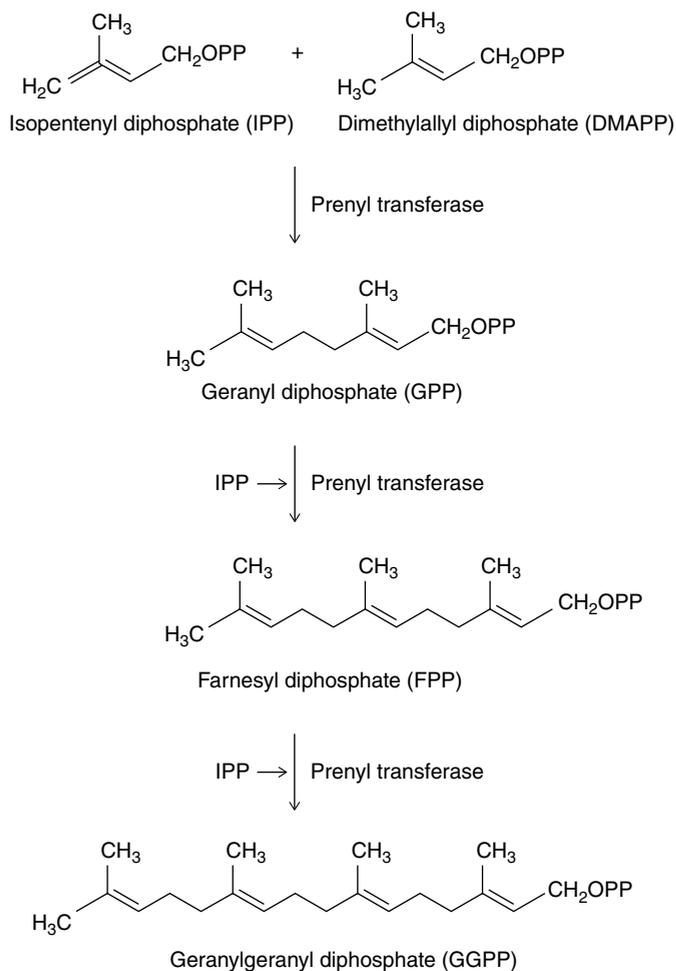


Figure 1.3 Synthesis of geranylgeranyl diphosphate (GGPP). From Thayumanavan & Sadasivam (2003).

terpenoid synthetic genes, that is, terpenoid synthases, P450, and so on, continue to be identified (Daviet & Schalk, 2010; Daniel, 2006; Muñoz-Bertomeu *et al.*, 2007; Muñoz-Bertomeu *et al.*, 2006; Wink, 1987).

The production of aroma relies on the genetic consideration and also on the growth phase of herbs. Other important factor is the environmental impact which could transform biochemically and physiologically those herbs modifying the amount and the constituents of the aroma. For this reason, the biotechnological formation of natural aroma compounds is speedily expanding although the conventional pathways of chemical reactions or removal from herbs are yet feasible. Terpenes are more costly to produce in relation to other metabolites due to the complexity of reactions. Since the advent of common food in the human life, such as beer, bread, yogurt, cheese, soy derivatives, wine and other fermented foods, microbial reactions have normally take part an important act in the production of

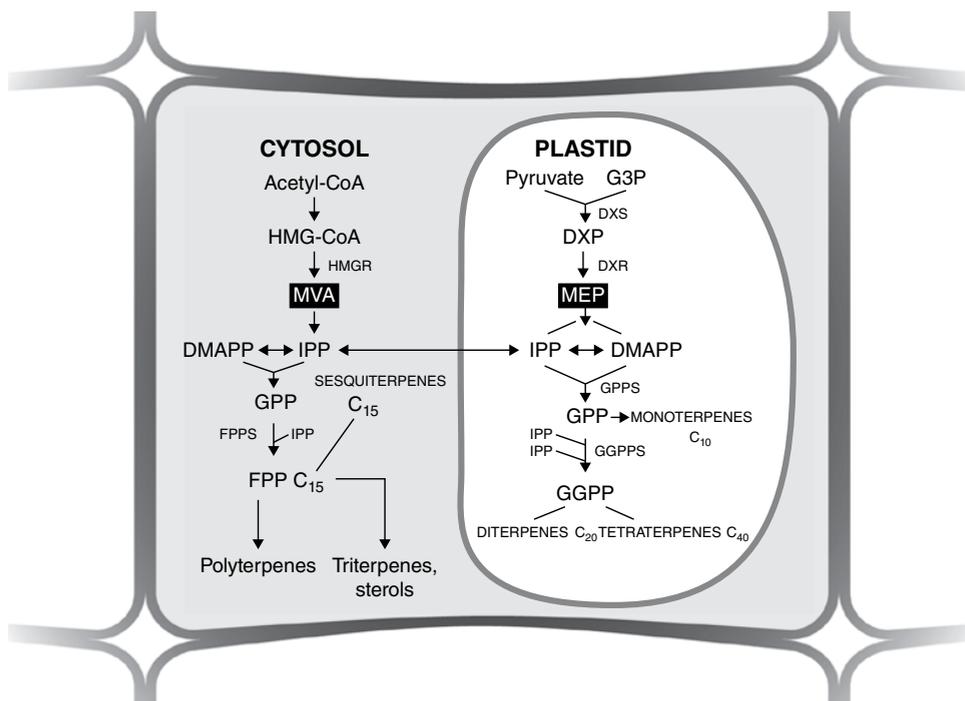


Figure 1.4 Enzymes involved in isoprenoid biosynthesis through cytosol (MVA) and plastids pathway (MEP): FPPS, farnesyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; GPPS, GPP synthase; and HMGR, 3-hydroxy-3-methylglutaryl CoA reductase. FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; G3P, D-glyceraldehyde 3-P; and HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

complex mixtures of food aromas. This background of biotechnology in our days has evolved from craft origins into big, attention-getting industries. Starting at the beginning of the last decade, the renewal of some techniques in the volatiles analysis area facilitated the separation and structural identification of essential oils, such as gas chromatography. There is no doubt that the use of molecular biological strategy is helping our comprehension of some herb metabolites and how they are used in the area of physiology. Monoterpenes, which are widely distributed in nature with around 400 structures, constitutes a satisfactory precursor basis. Transformations of composition and the amount from industrial production of aroma area by genetic engineering should have an impact in the commercial sector. In recent years, substantial progress has been made in biotechnology and in the genetic area; in particular, the progress of the molecular biology apparatus has been used to discover a lot of biosynthetic routes. The combination of this expertise with the apparatus accessible for the genetic area and metabolic engineering nowadays opens a large possibility to obtain new pathways for the generation of herbal compounds. This approach has been mainly applied to the obtention of high-value pharmacological actives (Prins *et al.*, 2010; Muñoz-Bertomeu *et al.*, 2007; Krings & Berger, 1998; Rhodes, 1994; Gershenzon, 1994).

1.1.2 Factors Influencing the Quantity and Quality of Essential Oil in Plants

The essential oils market is in constant expansion, moving tens of millions of dollars annually (Marques *et al.*, 2012). The greatest producing countries are Brazil, India, China and Indonesia, and the greatest consumers of essential oils are the United States (40%), European Union (30%) and Japan (7%). In addition to its employ as the main raw material for aroma and fragrance, food and pharmaceutical industries (Prins *et al.*, 2010), essential oils have important biological activities that have been disclosed in recent years. However, as it seeks its practical application and the development of new natural drugs containing active compounds from essential oils, there is an urgent need to standardise the plant material source. For this to become achievable, it is necessary to know all factors that exert influence on the production of essential oils by plants (Prins *et al.*, 2010).

Medicinal and aromatic plants produce essential oils as secondary metabolites in response to a physiological stress, pathogen attack and ecological factors. The stress caused by physical and chemical environmental conditions, for example, may exert great influence in plant's capacity to produce these metabolites. As regards the interactions of the plant-pathogen, essential oils are recognised as defence compounds that confer protection against several natural enemies, but they also facilitate the reproduction of the vegetal species by attracting pollinators (Prins *et al.*, 2010; Marques *et al.*, 2012). Thus, there has been suggested a dual role of these compounds on alleviating both environmental and pathogen attack stresses (Tezara *et al.*, 2014).

In order to optimise its commercial exploitation, the different factors involved in the production of essential oils must be taken into account, since the induction into its substances synthesis could affect the specific compounds of interest and their economic applications, besides affecting the quality and quantity of produced oil.

The main factors related to the essential oils production are discussed in the following sections.

1.1.3 Pathogens Attack

It has been demonstrated that volatile compounds operate as plant defence against animals, microorganisms and insect herbivores (Koeduka *et al.*, 2006; Sardans *et al.*, 2010; Fürstenberg-Hägg *et al.*, 2013; Cabral *et al.*, 2013). The plant-insect interactions have led the plants to develop various defence strategies against insect feeding (Paré & Tumlinson, 1999; Fürstenberg-Hägg *et al.*, 2013). These defence mechanisms can be constitutive or inducible. Some internal signals from affected tissues of the plant include calcium signalling, enzymes phosphorylation and jasmonate signalling pathway. These affected tissues and reinforce the production of low molecular weight compounds, such as the substances found in the essential oils, which are bioactive insect repellents or intoxicants. Still, these released volatiles attract several kinds of predators, through the connection from leaves or plants and to induce protection mechanisms. Moreover, a great number of these substances are produced to avoid future attacks.

The plants can release more than 1000 volatile organic compounds mainly consisting of 6-carbon aldehydes, alcohols, esters and several terpenoids from their different parts (Gobbo-Neto & Lopes, 2007; Fürstenberg-Hägg *et al.*, 2013). A curious fact is the way

by which the plant responds to pathogen attack, producing larger amounts of a specific compound that may also vary depending on the kind or specie of the intruder, or on the plant part invaded. Valladares *et al.* (2002) analysed the alterations in composition of the essential oils and volatile release from *Minthostachys mollis* (Kunth) started by two types of herbivorous insects — a leaf miner and a gall insect. The authors observed a reduction in the pulegone concentration that was associated with both kinds of insect damage, while the menthone content significantly increased only in mint leaves. It was also verified by Huang *et al.* (2012) that the majority volatile compound emitted from *Arabidopsis thaliana* (L.) Heynh. flowers, the (E)- β -caryophyllene is synthesised as a defence against *Pseudomonas syringae* pv. tomato, a pathogenic bacterium of brassicaceous plants. In these cases, the compound, (E)- β -caryophyllene, appears to serve as defence against pathogens that attack floral tissues and, like other floral volatile compounds, can have multiple roles in plant protection and pollinator attraction. Still, according to authors, flowers have a high risk of pathogen attack because of their rich nutrient and moisture content, and high frequency of insect visitors.

1.1.4 Environmental Factors

As mentioned before, several physical and chemical environmental factors can affect the quality and quantity of essential oils produced by medicinal and aromatic plants. Amongst these factors cited are the temperature, hydric and osmotic stress, relative humidity, photoperiod (light), nutrition (fertilisation), seasonality, soil properties (salinity, pH, chemical composition, toxins), genetic characteristics and harvest time (Abdelmajeed *et al.*, 2013). The expression of plants' secondary metabolism is also an answer to mechanical factors such as injuries, rain, hailstones, wind and sandstorms (Gobbo-Neto & Lopes, 2007; Fürstenberg-Hägg *et al.*, 2013).

Studies focusing on the effects of temperature on the production of essential oils by aromatic plants have shown that variations throughout the year, month or even day exert great influence in the plant development and hence affect the secondary metabolite production. In general, the production of essential oils increases at elevated temperatures, although it can lead to an excessive loss of these metabolites on very hot days (Lima *et al.*, 2003; Gobbo-Neto & Lopes, 2007). However, it has also been observed in a mobilisation and accumulation of certain plant metabolites in *Zea mays* L. (Christie *et al.*, 1994; Gouinguéné & Turlings, 2002), *Artemisia annua* L. (Wallaart *et al.*, 1999) and *Nicotiana tabacum* L. (Koeppel *et al.*, 1970) after submission at very low temperatures.

Although several studies approach the effects of temperature on the morphology and oil yield in plants, little information is available in literature about its effects in the volatile oil composition. Chang *et al.* (2005) observed an increase of three times on the levels of volatile oil in fresh leaves of *Ocimum basilicum* L. (basil) grown for two weeks at 25°C or 30°C, against leaves of plant cultivated at 15°C. The different temperatures also altered the chemical composition of the compounds present in the leaves. At 25°C there was an eugenol and *cis*-ocimene cumulation, whereas at 15°C a highest content of camphor and *trans*-farnesene was observed. In coriander, the highest oil percentage was also detected under stress temperature (Farahani *et al.*, 2008).

However, even small temperature increase of two to three degrees during the day could significantly raise the levels of essential oil in *Mentha piperita* L. (Bernáth, 1992).

The temperature effects on the production of secondary metabolites are also related to other factors, as latitude and seasonality. Seasonal fluctuations in the content of the different secondary compounds produced by medicinal and aromatic plants, including essential oils were reported. The seasonality, in turn, has a direct influence on the biomass production since vegetative development and foliar biomass are the key points for essential oil production and aromatic plant harvest (Marques *et al.*, 2012). Thus, in order to achieve highest essential oils yields, it is suggested that the harvest must be performed in the season in which higher essential oil contents are observed.

Silva *et al.* (2005) evaluated the effects of harvest season on the production and analytes present in the essential oil of *Ocimum basilicum* L. (basil) during six months. Higher yield in essential oil was obtained in January (summer – 2.26%) than in August (winter – 1.06%). However, a decrease of linalool was observed in the oil obtained in January. Alterations in the levels of the majority compounds of *Achyrocline satureioides* (Lam.) DC. (Macela), that is, trans-pinocaraveil, mistenil and β -pinene acetates were also observed for the oil derived from the floral capitulum originating at several harvest times (Bezerra *et al.*, 2008). Previous studies also have reported differences in the levels of essential oils obtained in different seasons. From a study about the composition and yield of the oil from *Salvia officinalis* L. grown spontaneously in Dalmatian Island, it was observed that the yield of the majority compounds, tujone, 1,8-cineole and camphor varied greatly. The amount of essential oil was higher in July, while the production of tujone was increased in October (Pitaveric *et al.*, 1984). Clones from this species on cultivate conditions produced maximum essential oil yield and high content of tujone in July, season of great luminosity (Putievsky *et al.*, 1992). On the other hand, in a study about the seasonal evolution in the oil composition of *Virola surinamensis* (Rol. ex Rottb.) Warb., no variation on oil yield was observed in the different seasons and times of harvest evaluated, but the relative percentage of the compounds was significantly altered (Lopes *et al.*, 1997).

The effects of day length and photon flux density on *Menthapiperita* L. oil composition have been studied by several authors in order to differentiate between photoperiodic and photosynthetic influences (Voirin *et al.*, 1990). Some authors (Grahle & Holtzel, 1963; Clark & Menary, 1979) concluded that 'the effects were caused by photoperiodic treatment rather than by differences in photosynthesate between short day and long day conditions'. Also, was observed a relation between daytime photosynthesis and nighttime utilisation of photosynthesate, which was directly influenced by the oil composition and the main interacting factor was the temperature (Burbott & Loomis, 1967).

In order to vouch essential oil yield and quality, it is very important to determine the environmental conditions and process factors that affect its composition. In the case of the essential oil from *O. basilicum* L. (basil), which is commonly used as flavouring and in cosmetics due to its high concentration in linalool and its value in the international market, the effects of harvesting season, temperature and drying period on its yield and chemical composition have been investigated (Carvalho-Filho *et al.*, 2006). Harvestings performed at 40 and 93 days after transplanting of seedlings showed higher essential oil yield when were carried out at 8 a.m. and at noon. After drying for a period of five days, the contents of linalool increased from 45.18% to 86.80%. These findings indicate that the ideal conditions for *O. basilicum* L. harvest is during morning and the biomass drying at 40 °C during five days aiming to get linalool-rich essential oil.

During the investigation of the daytime variation of the essential oil of four medicinal species in the central region of Iran, the yield of the essential oil from the *Eucalyptus nicholii* Maiden and Blakely leaves, *Rosmarinus officinalis* L., *Thuja occidentalis* L. and *Chamaecyparis lawsoniana* (A. Murray) Parl. also shows seasonal and diurnal variation if obtained at 7 a.m., at noon or at 6 p.m. (Ramezani *et al.*, 2009) which reinforces the conclusion that for obtaining the highest yields of essential oil and other volatile compounds, harvesting of plant material must be accomplished at special time during the day.

Sandeep *et al.* (2015) also emphasised the need for standardising and control of environmental and ecological factors for optimisation of plant compounds production. The secondary metabolites of turmeric (*Curcuma longa* L. cv. Roma) such a sessential oil, oleoresin and curcumin are used for multipurposes in medicine, cosmetics food flavouring and textile industries. The curcumin content may vary from place to place as function of environment, soil and agro-climatic conditions. Plants cultivated in several agro-climatic regions showed a variation of 1.4% to 5% of curcumin. The variation in the majority compounds of the essential oil, that is, tumerone and α -phellantrene in all the areas were about 10% to 20%.

The effects of environment conditions on the content of main metabolites may improve crop yield and quality of production, as demonstrated for numerous plant species (Formisano *et al.*, 2015). *Matricaria chamomilla* L. (german chamomile) is produced by large-scale cropping due to its medicinal and industrial importance. A study about the effects of environmental conditions on crop yield and on the chemical profile of the essential oils of different chamomile genotypes, which were cultivated in Molise (Italy) in different growing environments reveled that the crop yield was strongly influenced by the soil conditions and climate, and mainly by the altitude, fertility and the water supply increasing the chamomile productivity. Still, the chemical analysis of the essential oils shows the compounds *scis*-tonghaosu, spathulenol, α -bisabolol oxide B and α -bisabolol oxide A as main constituents in all samples, but their amounts in each plant varied significantly (Formisano *et al.*, 2015).

The market of the lavender essential oils is still increasing; however, it is not well understood how the environment and growth conditions can affect the ideal harvest period of its flowers for essential oil obtainment. Hassiotisa *et al.* (2014) evaluated how the essential oil quality and quantity of *Lavandula angustifolia* Mill. cv. Etherio can be influenced during blooming by environmental conditions. The authors investigated the relationship between the lavender essential oil production and the gene expression during blooming, aiming to determine the optimum period for essential oil harvest. The authors verified that essential oil content was regulated positively by temperature and flowering stage, while it was negatively influenced by pluviosity during the flowering period. The desired profile of the essential oil with respect to the content of linalool was influenced by temperature, flower development, LaLINSgene expression (involved in the biosynthesis of essential oil) and pluviosity. Although pluviosity has significantly decreased the linalool content, the standard quality of lavender essential oil has been normalised after 10 days, suggesting that linalool productionis regulated during blooming period for both environmental and growth factors. It was also proposed that the optimum harvest time to obtain rich essential oil is when the lavender reaches 60% of blooming, it is over 26°C and that it does not rain for a period of 10 days before harvesting.

1.1.5 Hydric Stress

Water is essential for life and for the metabolism of plants. So it would be logical to assume that the production of secondary metabolites would be higher in humid environments. However, this does not always occur (Morais, 2009). The hydric factor significantly impacts the growth evolution of the whole plant, and its frequency and intensity are elements of great importance for the limitation of global agricultural production (Ortolani & Camargo, 1987). Many physiological factors such as opening and closing of stomata, photosynthesis, growth and leaf expansion may change when the plant undergoes drought stress, which can cause alterations in the secondary metabolism.

According to Abdelmajeed *et al.* (2013), drought stress is induced by a restriction of the water supply that affects the leaf water potential and turgor decrease, stomata shutdown and a decrease in cell extension and growth. The hydric stress can decrease the relative plant water content from 77.7% (Rahbariana *et al.*, 2010). The decrease in plant development is a consequence of its impact on different physiological and biochemical processes, such as photosynthesis, respiratory chain, translocation, ion uptake, carbohydrates, nutrient metabolism and growth promoters.

The excess of water on the ground can change chemical and biological processes, limiting the amount of oxygen and accelerating the formation of toxic compounds to roots. On the other hand, severe percolation of water causes the removal of nutrients and inhibiting the normal growth of the plant. Water surplus, while important, causes fewer problems than the dry periods (Ortolani & Camargo, 1987). Several authors reported an increase in certain plant metabolites under hydric stress conditions, mainly the content of terpenoids (Ortolani & Camargo, 1987). The increase of terpenoids under water stress seems to occur due to a low allocation of carbon to the plant development, suggesting a trade-off between growth and defence mechanisms (Turtola *et al.*, 2003). Under moderate and several water stress, an increase in thymol content was observed in origanum (Bahreininejad *et al.*, 2013). Furthermore, it was reported for three different origanum varieties that the water deficit after early flowering (double flowers) can lead to an increase in the essential oil levels and may result in a higher quality of origanum plants and the highest water recovery effectiveness by this plant (Aziz *et al.*, 2008).

Important reductions in the growth parameters under water deficit, such as vegetative growth, lipids content and essential oil level were observed in *Salvia officinalis* L. (Belaqziz *et al.*, 2009) and *Cuminum cyminum* L. (Rajeswara, 2002). The results suggested that water shortage might act in the regulation of the production of bioactive constituents in cumin seeds, interfering in their nutritional and economic values.

In chamomile, the effects of drought stress were decreasing of plant size, number of flowers, shoot weight and apigenin level, but did not affect the essential oil quantity or its composition (Baghalian *et al.*, 2011). Also, water stress caused a significant decrease in all development parameters of *Mentha piperita* L. and in turn the essential oil productivity. Highest levels of menthol were achieved under 70% of biomass production capability (Farahani *et al.*, 2009). On the other hand, the drought influenced the essential oil content in *Nepeta cataria* L. (lemon catmint) and *Melissa officinalis* L. (lemon balm), resulting higher amount of essential oils (Iness *et al.*, 2012).

Another important factor that can change the yield and chemical composition of essential oils is the rainfall (Morais, 2009). Constant, intermittent rain can result in the loss of hydrosoluble substances mainly from leaves and flowers. It is recommended to wait about three days after the end of the rains for the plant collection, so that the essential oil levels return to normal.

Therefore, hydric stress, that is, water deficit or excess is one of the most important factors involved in the synthesis of essential oils by medicinal and aromatic plants, since they can alter significantly the plant growth, as well as to modify the composition and concentration of metabolites present in the essential oils, being the major cause of lost productivity; it is directly correlated with the secondary metabolites concentration.

1.1.6 Plant Nutrition

According to Marques *et al.* (2012), amongst all the factors influencing plant activities, nutrition requires the greatest attention since the excess or lack of nutrients may be directly related to the variations in the active substances production. This also depends on the interaction with other factors such as water availability and soil physical properties.

An increase in the synthesis of essential oils as the increase at nitrogen doses, between 3.4–13.8 kg.ha⁻¹, have been reported for three *O. basilicum* L. (basil) cultivars (Sifola & Barbieri, 2006). However, the differences in relation to the oil quality amongst the plants appear to be unrelated to the supplies containing nitrogen. On the other hand, some authors observed that formulas containing organic and inorganic N sources allowed optimal plant performance and have incurred higher yields in essential oils. Still, the mixtures altered the chemical composition of the essential oils, decreasing the amount of linalool and increasing the methyl chavicol content (Singh *et al.*, 1991).

Teles *et al.* (2014a) observed that plant nutrition did not impact the biomass and essential oil synthesis of *L. origanoides* Kunth plants. However, some changes have been observed in the essential oils composition, as well as changes in the antioxidant activity of the oils. It was concluded that for growth and essential oil production, additional fertilisation are not required for *L. origanoides* at the employed conditions; however, in order to increase the biological activities of this essential oil, organic fertilisation is suggested. After the authors studied the chemical composition, antioxidant activity and production of essential oils from *L. origanoides* Kunth plants cultivated under organic and mineral supplementation, and harvested at different development stages (Teles *et al.*, 2014b). The production and composition of essential oils were not affected by fertilisation, but their composition and antioxidant activity were influenced by the plant age, being that essential oils from grown plants showed lower antioxidant activity than young plants. In the other hand, the essential oil produced from organic fertilisation showed better antioxidant potential when compared with mineral fertilisation. In conclusion, these results show the potentialities and properties of *L. origanoides* essential oil in specific conditions as showed before indication that early harvest of and organic nutrients could be applied to increase plant activities.

Pelargonium graveolens L'Her., a significant economic crop and source of geranium essential oil have been studied as to answer to nutrient handling on culture productivity, biochemical parameters, essential oil production and profile, nutrient content and

antioxidant activity during 2012 and 2014 in Lucknow, India (Pandey & Patra, 2015). The use of different blends from poultry manure as organic fertiliser plus a chemical treatment were able to enhance plant stature, leaf area, essential oil yield and antioxidant activity when compared to control. Besides, the percentage of the majority oil compounds increased in the blend application of organic and chemical fertilisers. These results allowed to conclude that combined application of 50% of each nutrient kind (75:30:30 N:P:K kg ha⁻¹ and 2.5 kg ha⁻¹ poultry manure) incurred in an important increase of the plant growth parameters and oil yield with better flavour profile, enhanced antioxidant activities and improvement of soil characteristics.

1.1.7 Genetic Factors and Chemical Diversity

As previously mentioned, the total content as well as the relative ratios of essential oils in plants may vary depending on various factors, including the stage of plant development. Although a genetic control for the expression, this may also be influenced, according to Marques *et al.* (2012) by modifications resulting from the interactions of biochemical, physiological, ecological and evolutionary processes. Generally, the chemical diversity of secondary compounds does not only occur between and within plant families and genera but also within populations of a single species, forming chemotypes (Kleine & Müller, 2011). Thus, the synthesis of secondary metabolites in populations of the same species can vary.

In one of the pioneering works, the content and constitution of oils of 10 peppermint genotypes were analysed during three different years (Gasic *et al.*, 1987). The parameters varied both in relation to year of investigation and the genotypes assayed and the genotypes showed a specific response to environmental conditions.

More recently, several studies have focused on the geographic region and chemical variety of plant populations, but whether this structure conforms to a central-marginal model or a mosaic pattern has not been elucidated (Bravo-Monzón *et al.*, 2014). According to Bravo-Monzón *et al.* (2014), the evaluation of the chemical variety of weeds in their native habitats facilitates the knowledge of their relationships with natural predators. Thus, the authors evaluated the geographic fluctuation of diversity in Mexican populations of the bittervine weed *Mikania micrantha* Kunth and its relationship to herbivore damage. A stepwise multiple regression analysis was used to establish a relationship between geographic, climatic and chemical diversity variables and damage by herbivores. However, population-level chemical diversity was the only significant variable. The authors concluded that fluctuations in chemical diversity follow a mosaic pattern in which geographic factors or natural enemies present some effect and that is also correlated with the predators' attack.

The geographic difference had a important effect on the oil concentration in 12 genotypes or ecotypes of *Zataria multiflora* Boiss. from several regions (Sadegui *et al.*, 2015). This plant is popularly used as a spice in Iran. Three ecotypes show maximum oil yield, with a maximum difference of 1.09% between the populations. The determination of the main compounds in relation to quantity of essential oil components have allowed the recognition of three chemotypes: carvacrol, thymol and linalool of which the thymol chemotype is the most common in different parts of Iran. The chemical variability was related to genetic and environmental factors.

In accordance with Tezara *et al.* (2014), differences in terpene production amongst three chemotypes of *Lippia graveolens* Kunth. (Mexican oregano) growing wild (rainy season) and in a common garden in the Yucatán peninsula were generally unrelated to either site of origin, rainfall or chemotype, although amongst the four populations selected for physiological measurements in the field, the T (tymol) populations produced more terpene than the S (sesquiterpenoids) populations. The highest terpene content was found in plants from the high-rainfall T population with the highest photosynthetic rate. Terpene content, which was higher in plants sampled in the field than in the common garden, was determined by photosynthetic rate and water use efficiency, and only by photosynthetic rate in the garden. According to the authors, the underlying reasons for the difference between the results obtained in the field and common garden are apparently not microclimatic.

Knowing the variations in the chemical compositions of different genotypes of a plant is of commercial importance as we seek to use them in different industrial segments.

1.2 Conclusions

The present chapter shows that through knowledge of the chemical characteristics and the main factors involved in the production of essential oils by plants, it is possible to standardise the plant material, and therefore the essential oil composition. This is primordial to achieve adequate quantity and quality, mainly in terms of chemical oil composition, in order to its employment for commercial purposes and supply market demands.

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2

Extraction Methods of Essential Oils From Herbs and Spices

Shahin Roohinejad^{1,2}, Mohamed Koubaa³, Francisco J. Barba⁴,
Sze Ying Leong^{5,6}, Anissa Khelifa³, Ralf Greiner¹ and Farid Chemat⁷

¹ Department of Food Technology and Bioprocess Engineering, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Germany

² Burn and Wound Healing Research Centre, Division of Food and Nutrition, Shiraz University of Medical Sciences, Iran

³ Sorbonne Universités, Université de Technologie de Compiègne, Laboratoire Transformations Intégrées de la Matière Renouvelable, France

⁴ Universitat de València, Faculty of Pharmacy, Nutrition and Food Science Area, València, Spain

⁵ Department of Food Science, University of Otago, New Zealand

⁶ Department of Safety and Quality of Fruit and Vegetables, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Germany

⁷ Université d'Avignon et des Pays de Vaucluse, France

2.1 Introduction

Essential oils (EOs) represent a group of volatile aromatic compounds that are synthesised by different plant species. These molecules have been used for many years as flavouring agents for the preparation of food and cosmetic products, as well as in the traditional medicines for therapeutic purposes (Burt, 2004; Hussain *et al.*, 2008; Valgimigli, 2012; Teixeira *et al.*, 2013). In fact, numerous pharmaceutical and biological activities, such as antibacterial (Burt, 2004), antifungal (Tabassum & Vidyasagar, 2013), insecticidal (Zoubiri & Baaliouamer, 2014), anticancer (Bayala *et al.*, 2014), antimutagenic (Raut & Karuppayil, 2014), antiviral (Astani *et al.*, 2011), antidiabetic (Yen *et al.*, 2015) and antioxidant and anti-inflammatory (Miguel, 2010) activities, have been associated with EOs. Therefore, due to all of these beneficial properties, they have attracted great interest in both scientific and industrial communities.

EOs are generally liquid at room temperature. Their solubility in water is low, compared to that in fats, alcohols, organic solvents and other hydrophobic solutions (Thormar, 2011). In this respect, numerous extraction methods have been developed to recover these molecules from plant materials. Although steam- and hydrodistillation are the most applied methods at commercial scale for the extraction of EOs (Burt, 2004; Lahlou, 2004), numerous novel extraction technologies (*e.g.*, supercritical fluid extraction; Pourmortazavi & Hajimirsadeghi, 2007), ultrasound- (Vinatoru, 2001) and microwave-assisted extraction (Cardoso-Ugarte *et al.*, 2013), known also as non-conventional methods, have been developed as alternatives to traditional methods, in order to meet

Table 2.1 Characteristics, main disadvantages and advantages of various processes for essential oils extraction.

Name	Investment	Sample size	Extraction time	Main disadvantages	Main advantages
Hydrodistillation or steam distillation	Low	>1000L	High	Limited by temperature	Large scale
Solvent extraction	Low	>1000L	High	Limited by solubility	Large scale
Ultrasound	Low	600L	Low	Problem for separation	High-cell disruption
Microwave	Medium	150L	Low	Hot spots	Cell disruption
Ohmic heating	Medium	Continuous	Low	Need of know-how	High-cell disruption
SFE¹	High	300L	Medium	Need of know-how	Enhance mass transfer
PEF²	High	Continuous	Medium	Difficult ease of operation	Electroporation of wall cells

¹ SFE: Supercritical fluid extraction.

² PEF: Pulsed electric fields.

the green extraction concept for the recovery of natural products. Characteristics, main advantages and disadvantages of using different processes for extraction of EOs are shown in Table 2.1.

Numerous studies have described this concept as the methodology that allows either avoiding or minimising the use of organic solvents to extract high-added value compounds, along with other beneficial features including reducing the treatment time, intensifying the mass transfer phenomena, reducing the consumption of energy, preserving high quality of the extract and increasing the yields of extraction (Chemat *et al.*, 2012; Rombaut *et al.*, 2014). In addition, extraction of EOs by means of emerging technologies normally occurred at low and mild processing temperatures, which can preserve them from degradation, in contrast to steam- and hydrodistillation techniques. For this purpose, this chapter describes both conventional and non-conventional extraction methods of EOs from herbs and spices.

2.2 Conventional Methods of Extraction

Hydrodistillation, steam distillation and solvent extraction are the most applied conventional methods for the extraction of EOs from herbs and spices.

2.2.1 Hydrodistillation

Hydrodistillation (HD) is the most common method used for EOs extraction (Meyer-Warnod, 1984). In principle, it corresponds to a heterogeneous distillation. This method consists of immersing the plant material in a water bath; the mixture is then heated to

boiling point, at atmospheric pressure (Figure 2.1A). Under a source of heating, odorous molecules contained in the plant cells are released in the form of an azeotropic mixture. Although most of the components have boiling points above 100 °C, they are mechanically driven with the water vapor. The cooling by condensation leads to the separation of the mixture water and the EOs by decantation. The ‘Clevenger’ system advocated by the European Pharmacopoeia allows the recycling of the aqueous phase of the distillate in the boiler through a cohobage system (Clevenger, 1928). Thus, the water and the volatile molecules (EOs) are separated by their differences in density. The HD duration is generally between three to six hours depending on the plant material. This parameter can affect the yield of EO and its chemical composition.

HD is an effective, high-yield extraction method for herbs and spices in which the EOs are difficult to isolate, and particularly rich in non-water soluble and thermal stable

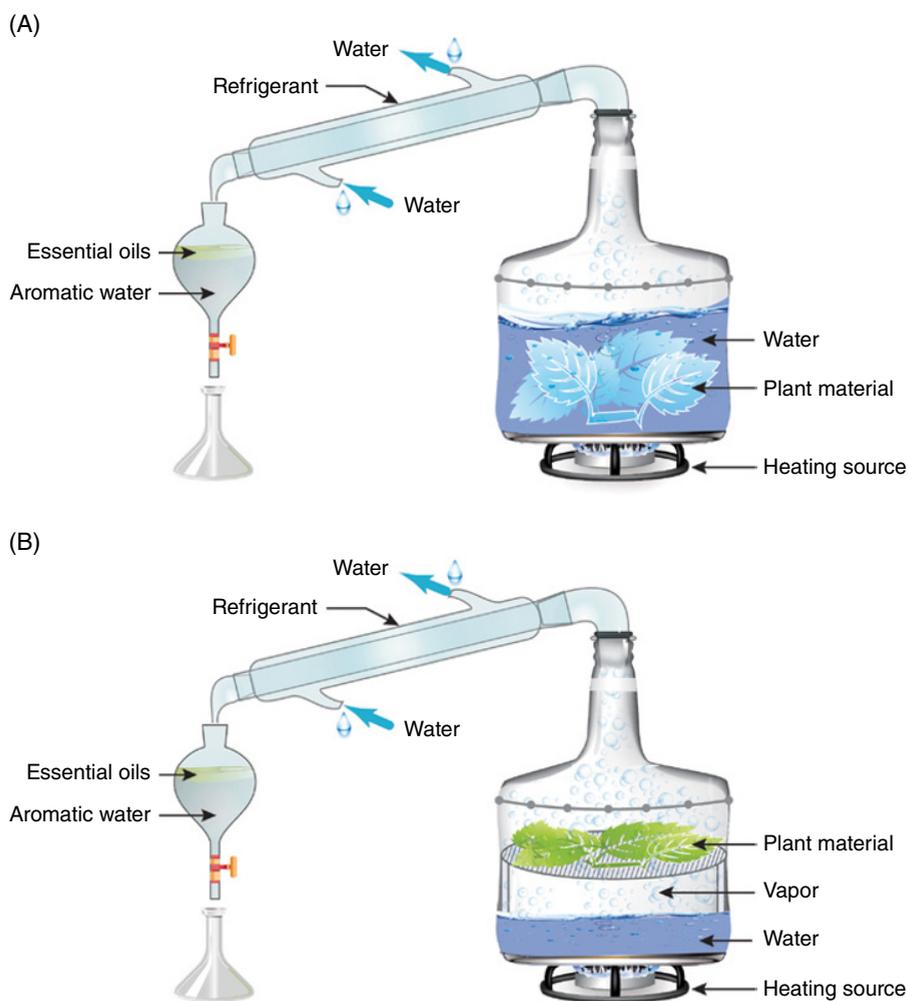


Figure 2.1 Schematic representation of hydrodistillation (A) and steam distillation set-up (B).

constituents that possess high boiling point. Otherwise, prolonged boiling of herbs and spices in water at high temperatures can promote hydrolysis of unsaturated or ester compounds, polymerisation of aldehydes or thermal decomposition of other heat-sensitive components that would reduce the overall quality of EOs (Gavahian *et al.*, 2012; Périno-Issartier *et al.*, 2013). On a positive note, one of the advantages of HD is that no chemical solvents are involved and hence the presence of toxic solvent residue in the EOs and losses of more volatile compounds during the subsequent removal of solvent can be avoided with HD (Filly *et al.*, 2016).

The yield and the exact composition of EOs may vary from one extraction variable (*e.g.*, plant-to-water ratio and heating duration) to another during HD, as well as affected by the status of raw material to be extracted (*e.g.*, plant species and plant parts developmental growth stages, seasonal variation, health status of plant, post-harvest treatment). Selected previous studies are presented below to provide an overview on the successful application of HD to guarantee a high extraction/recovery yield of EOs on different herbs and spices that would still retain many valuable constituents, by taking into account several plant and extraction condition factors.

- Oil yield and quality are highly species/cultivar dependent and differed across different plant parts. For different cultivars of basil, it was found that the extracted oil yield isolated by HD was the highest in flowers for *Ocimum basilicum* and in leaves for *Ocimum micranthum* (Charles & Simon, 1990). In another study, EOs of dill and caraways contained similar amount of carvone, but both the hydrodistilled EOs showed qualitative and quantitative differences in the minor constituents (Bailer *et al.*, 2001).
- The EOs content and composition after HD can be influenced by the plant growth stages of herbs and spices. It was found that EOs were better extracted from *Thymus kotschyanus* harvested at its complete flowering stage as compared to plants harvested before and at the beginning of flowering (Sefidkon *et al.*, 1999). Moreover, the hydrodistilled EOs of matured coriander (*Coriandrum sativum* L.) fruits were richer in monoterpene alcohols and ketones than those extracted from immature coriander fruits (Msaada *et al.*, 2007).
- Oil quality is also affected by seasonal variation. Basil harvested in the winter season gave the highest oil yield after HD while the least amount was obtained from basil harvested in summer (Hussain *et al.*, 2008). Owing to the difference in temperature, humidity and stage of plant metabolism over the year and season changes, winter basil oils were found to be enriched with oxygenated monoterpenes while summer basil oils were higher in sesquiterpene hydrocarbons. Likewise, the EO composition of *Aristolochia longa* ssp. *paucinervis* after HD varied considerably between harvest years from 2009 to 2013 as reported in a recent study (Dhouioui *et al.*, 2016).
- Changes in the quality of EOs extracted from herbs and spices cultivated were observed under extreme oxidative stress condition, for example, salt stress. Several herbs and spices such as basil (Tarchoune *et al.*, 2013), mint (Kasrati *et al.*, 2014; Yu *et al.*, 2015) and safflower (*Carthamus tinctorius* L.) (Harrathi *et al.*, 2011) were found to be salt-sensitive and therefore a reduction in oil yield was observed, followed by considerable changes in the chemical and volatile constituents of the EOs after HD. However, few studies have found that salinity may be beneficial to induce the biosynthesis of some important secondary metabolites in the EOs of sage (*Salvia officinalis* L.)

(Aziz *et al.*, 2013), of the Iranian spices *Salvia mirzayanii* (Valifard *et al.*, 2014), and of parsley (*Petroselinum crispum* ssp. *crispum* L.) (Aziz *et al.*, 2013) after being isolated using HD.

- Furthermore, changes in the oil yield and quality for herbs and spices pre-treated with different drying methods prior to HD were reported. The aerial parts of *Satureja hortensis*, an aromatic plant sharing the same family as well-known culinary herbs such as basil, mint and rosemary, were dried differently (under sun, under shade and in oven) and the highest oil yield and carvacrol was obtained from *Satureja hortensis* previously oven-dried at 45 °C (Sefidkon *et al.*, 2006). In another study, dried basil (25 to 30 °C) is reported to retain similar constituents in the hydrodistilled oils to that of fresh basil (Charles & Simon, 1990). A rather novel approach involving a combination of pre-drying by convection (40 °C) and drying with vacuum-microwave (240 W) was found to be a great option to dry thyme resulting in hydrodistilled oils of high sensory quality (Calín-Sánchez *et al.*, 2013).
- An effect of plant-to-water ratio during HD on oil yield was observed. The yield of EOs was not affected by the amount of herbs and spices used during HD, as demonstrated in the leaves and flowers of basil (1:100 versus 1:12.5 ratio) (Charles & Simon, 1990) and fennel seeds (1:4 versus 1:2 ratio) (Mimica-Dukić *et al.*, 2003).
- In addition, an effect of HD duration on the EOs composition was found. Prolonged HD (12 h versus 6 h) led to an additional 20% and 5% increase in the oil yields and phenylpropanoid content, respectively from fennel seeds (Mimica-Dukić *et al.*, 2003). The degree of degradation for some monoterpenes and oxygenated monoterpenes in fennel oil was found to be lower with longer HD duration. For *Ruta chalepensis* L., a perennial herb, it was found that 90 min of HD was adequate to recover nearly 80% of the major constituent in the EOs, that is, 2-undecanone when compared to 60 and 120 min of HD (Mejri *et al.*, 2010). In a recent study, the EOs from *Thymus pallescens* de Noé extracted for 30 min demonstrated the highest antioxidant, antimicrobial, and insecticidal activities (Benchabane *et al.*, 2015). For coriander oil, it is rather prominent to observe that the degree of recovery for different chemical constituents varied according to the HD duration, in which the low boiling point constituents (*e.g.*, α - and β -pinene, limonene, myrcene and γ -terpinene) achieved greater recovery within a short HD while the constituents with high boiling points (*e.g.*, geranyl-acetate and geraniol) required a long HD time for the greatest recovery (Zheljzkov *et al.*, 2014a).

In an attempt to reduce further the extraction time and improve the extraction efficiency, slight modifications on the existing HD have been recently explored, namely turbo-hydrodistillation, salt-hydrodistillation, enzyme-hydrodistillation and micelle-hydrodistillation. Turbo-HD was used for the first time in the extraction of EOs from the *Alliaceae* family (Mnayer *et al.*, 2015, 2014). At laboratory-scale level, this technique is similar to that of conventional HD, in which a stainless steel stirrer is installed into the flask of a Clevenger extractor to accelerate the extraction process. The stirrer is usually equipped with sharp blades that would cut/grind the plant material into small pieces during distillation to promote greater extraction of EOs. The resulting oils were found to be rich in total phenolic content and flavonoids while demonstrating strong antioxidant and antimicrobial activities (Mnayer *et al.*, 2015, 2014). Turbo-HD has also been used to extract EOs from flowers of lavender (Filly *et al.*, 2016). Although the extraction yield did not increase considerably to that of conventional HD, the major volatile

compounds of lavender EOs were higher, and when evaluated olfactorically, the aromatic lavender's notes namely 'floral lavender', 'fruity citrus/bergamot', 'camphoraceous' and 'herbaceous' were well identified by turbo-HD.

Addition of an appropriate 'additive' to the water for HD is advantageous to speed up the extraction process. For example, a small amount of salt (75 g NaCl per kg of plant) was added to the water before HD to raise the water's boiling point and thus the possibility to increase the oil yield (+42%) and the content of monoterpene alcohols (+26%) in the oils of damask rose (*Rosa damascene* Mill) flowers, as well as keeping the amount of hydrocarbons at low amount when compared to conventional HD alone (Kumar *et al.*, 2016). Similarly, Shamspur *et al.* (2012) observed the same results in a study on rose oil extraction. In lavenders, the use of salted water (150 g NaCl in 3 L water) during HD enhanced the oil yield and recovered some lavender constituents namely α -thujene, α -terpinene and geraniol that were not present in the oils extracted by conventional HD (Filly *et al.*, 2016). In contrast, salt addition in HD was not able to improve the extraction efficiency and oil yield from myrtle (*Myrtus communis* L.) (Gavahian *et al.*, 2013) and rosemary (Fazlali *et al.*, 2015). Instead of using sodium chloride to enhance the efficiency of HD, 1-hydroxyethyl-3-methylimidazolium chloride salt was added to the HD water. This ionic liquid additive was shown effective to increase the rosemary oil yield by 25% (Flamini *et al.*, 2015). Therefore, it is clear that the magnitude of the salt effect depends upon the type and the concentration of salt applied. Hassanpouraghdam *et al.* (2012) compared the effectiveness of sodium chloride, sodium carbonate, calcium chloride and calcium carbonate on the EO constituents and oil recovery from an herbaceous plant of *Mentha pulegium* L. They found that the chemical constituents of the oils varied significantly due to the addition of different salts during HD.

EOs are localised in the epidermal cells of flower petals, or accumulated in different plant anatomical parts, such as intracellularly secreting cells, glandular trichomes or secretory canals and pockets (Dima and Dima, 2015). Therefore, another efficient strategy to assist the conventional HD, in favouring the release of more EOs and their chemical and volatile components from their localisation in the plant cells, is through enzyme-mediated HD. Herbs and spices are pre-treated with enzymes, usually by using a single enzyme or several enzymes, such as cellulase, hemicellulase, xylanase, pectinase or protease, to induce partial or complete breakdown of the cell walls (Bhat, 2000; Kashyap *et al.*, 2001) before performing HD. The use of these enzymes to reduce the HD duration, to enhance the extraction yield of EOs, to promote the antioxidant and antimicrobial activities of EOs in bay leaves (Boulila *et al.*, 2015), lavender flowers (Filly *et al.*, 2016), rosemary (Hosni *et al.*, 2013), garlic (Sowbhagya *et al.*, 2009) and thyme (Hosni *et al.*, 2013) have been reported. However, it was observed that enzyme pre-treatment might lead to enhance the oxidation rate after the disruption of the cell walls. This would explain the detection of atypical off-notes in lavender oil (Filly *et al.*, 2016) after enzyme-mediated HD and in bay leaves, a remarkable increase in oxygenated monoterpenes and some losses of important volatile compounds were found (Boulila *et al.*, 2015). Taken together, a more thorough evaluation on the impact of an enzymatic pre-treatment, concerning the degradation of volatiles, on a range of different herbs and spices is needed before adopting this assisting technique alongside with HD.

Polysorbates (or commonly named as Tween) are surfactants and emulsifiers. Addition of polysorbates is amongst the other methods reported in the current

literature to complement the existing HD technique for the purpose to increase the extraction yield of EOs. Due to the amphiphilic properties of polysorbates, their presence in water during HD would promote formation of 'micelles' that simultaneously reduce the surface tension between the EOs of herbs and spices and the distillation water. To date, micelle-mediated extraction followed by conventional HD was first investigated in rose flowers (Baydar & Baydar, 2005). The recovery of rose oil by HD was performed in water containing Tween 20 ($C_{58}H_{114}O_{26}$) at various concentrations (0–5000 ppm). It was found that the addition of 2500 ppm of Tween 20 increased the oil yield by 50%, without the need to extend the HD duration to 12 h. Likewise, such increase in the oil yield due to micelle-HD was recently reported in lavender flowers extracted in water containing 10% (v/v) Tween 40 ($C_{62}H_{122}O_{26}$) (Filly *et al.*, 2016). The obtained lavender oils were enriched with chemical constituents including linalyl acetate, linalool, and 4-terpineol, which lead to a high intensity of perceived aroma of 'fatty/aldehyde', 'fruity citrus/bergamot' and 'sweet/candy'.

2.2.2 Steam Distillation

Steam distillation (SD) is one of the ancestral methods applied for the recovery of EOs. It is also amongst the best-suited methods to obtain high quality of EOs. For the past decades, large quantities of the EOs of herbs and spices for commercial use have been usually extracted by SD (Burt, 2004). SD is suitable to extract heat sensitive components (*e.g.*, volatiles), and purify them by the application of steam. Distilled water is used as the steam source for this technique. The extraction process works in a way that the plant material containing the compounds of interest is distilled at a temperature below their boiling point and the application of steam would volatilise them at a temperature lower than 100°C, at atmospheric pressure (Rojas & Buitrago, 2015). The volatile compounds are driven by the steam, which passes through the aromatic plant from the bottom of the alembic to the top (Figure 2.1B). The steam destroys the structure of plant cells, releases the contained molecules and takes away most of the volatile components. The oil is then carried by the steam out of the column and into a cooling system (condenser) where the steam is condensed back to a mixture of water-oil liquid. This mixture is then collected in a receiving vessel. The removal of water layer, referred to as a 'hydrosol', from the distillate is generally required after distillation and once there is no more EOs that could be obtained, these water and oil layers are then separated by decantation in a vessel usually called 'Florentine flask' or 'separatory funnel' (Dugo & Di Giacomo, 2002). Alternatively, the EOs of the volatile distillate are directly collected from the top of the hydrosol or the water in which the EOs are collected, is removed by evaporation.

As high temperature is involved during SD, chemical alteration in the compounds of interest cannot be avoided (Pecorino-Issartier *et al.*, 2013). A number of factors determine the final quality of steam-distilled EOs from herbs and spices. One of them is related to the plant material itself such as the variations between species, parts of plant, individuals, stress conditions, seasons and harvesting areas (Baher *et al.*, 2002; El Asbahani *et al.*, 2015; Gilles *et al.*, 2010; Parejo *et al.*, 2002; Perry *et al.*, 1999; Yildirim *et al.*, 2004; Zheljzakov *et al.*, 2008). A careful control of the steam distillation duration and temperature is important too. In recent years, extensive experiments have been conducted to assess the EO yields and compositions from a wide range of herbs and

spices as a function of the SD duration. For instance, SD performed for merely 20 min was shown to promote a maximum increase in EO yields of peppermint (1.89% oil yield), lemongrass (0.163% oil yield) and palmarosa (0.182% oil yield), and prolonged SD (up to 160 and 240 min) was not shown to further increase the yield of EOs (Cannon *et al.*, 2013). The optimum SD duration to ensure efficient oil extraction varied between different plant materials. For instance, it was demonstrated that the yield of EOs reached a maximum after 10 min for Japanese corn mint (1.04%) (Zheljazkov & Astatkie, 2012), 60 min for lavender (6.80%) (Zheljazkov *et al.*, 2013d), 160 min for pine (0.10%), fennel (0.68%) and sweet sage wort (0.23%) (Zheljazkov *et al.*, 2013a, 2013e, 2012b), 240 min for oregano (2.32%) (Zheljazkov *et al.*, 2012c), 360 min for anise (2%) (Zheljazkov *et al.*, 2013c), and 480 min are needed to obtain a sufficient oil yield from chamomile (0.28%) by SD (Gawde *et al.*, 2014).

Table 2.2 summarises the impact of varying SD duration on the oil yield and composition from a wide range of herbs and spices in the current literature. It is clear that SD duration greatly affected the oil yield and composition and the maximum yields achieved for each individual chemical constituent in the EOs is highly dependent on their thermal stability properties. It is rather conclusive that the yield of low-boiling point EO constituents are always higher at shorter SD time and degraded with extended SD time, while a reversed trend is observed for the high-boiling point constituents (Zheljazkov *et al.*, 2014b, 2013a, 2012a). For commercial implication, this suggests the importance of distillation duration to possibly tailor EOs with targeted composition to meet certain needs such as enhanced biological activities and therapeutic values due to the maximum recovery of specific desirable chemical constituents. In other words, desirable chemical constituents in EOs would be preserved and the generation of undesirable constituents can also be minimised under optimum length of SD process, in order to produce tailored EOs for specific market uses.

Improvement of steam-distilled EOs by several preliminary maceration treatment, namely microwave heating (Sahraoui *et al.*, 2008; Sahraoui & Boutekedjiret, 2015) and addition of cell-wall degrading enzymes (Sowbhagya *et al.*, 2011, 2010), to assist the disruption of plant cell structure in order to release more EOs and their chemical and volatile components have been evidenced. These pre-treatments on plant samples were shown promising to increase the efficiency of SD, reduce the energy uses and produce high yield and quality of EOs. The merits of these novel extraction strategies will be further elaborated in the subsequent sections of this chapter.

2.2.3 Solvent Extraction

Solvent extraction (SE) involves dissolving the plant material that contains EOs in a solvent and then evaporating it to recover the EOs. After extraction, the liquid mixture containing the EOs (and other compounds) passes through a filtration process and an ulterior distillation. Because of its high volatility, the most frequently used solvent is hexane. Benzene and dichloromethane are also commonly used but have been banned because of their toxicity. Due to the diversity of chemical families in EOs that can be extracted with solvent extraction, this method has many applications in the field of agribusiness (Jadhav *et al.*, 2009). For instance, Bimkr *et al.* (2011) have investigated the potential use of solvent-assisted extraction of flavonoids contained in green mint (*Mentha spicata* L.).

Table 2.2 Effect of different steam distillation (SD) durations on the essential oil composition.

Herbs and spices	SD time (minute)	Maximum recovery of the desired chemical constituents in the essential oils after SD*	References
Japanese corn mint (<i>Mentha canadensis</i> L.)	1.25	α -pinene Sabinene β -pinene 3-octanal Limonene Eucalyptol Isopulegon Menthone Isomenthone β -bourbonene	Zheljazkov & Astatkie (2012)
	160	Menthol	
<i>Juniperus scopulorum</i> trees	1.25	α -thujene α -pinene Sabinene <i>p</i> -cymene Camphene	Zheljazkov <i>et al.</i> (2013b; 2012a)
	5	Myrcene	
	40	<i>cis</i> -sabinene hydrate Linalool	
	480	Limonene 4-terpinenol δ -cadinene Elemol	
	720	α -terpinene γ -terpinene Terpinolene	
Oregano (<i>Origanum vulgare</i> L.)	1.25	α -thujene α -pinene Camphene Myrcene α -terpinene <i>p</i> -cymene Limonene γ -terpinene <i>cis</i> -sabinene hydrate Terpinolene	Zheljazkov <i>et al.</i> (2012c)
	10	Borneol 4-terpineol β -bisabolene β -caryophyllene	
	40	Carvacrol	

(Continued)

Table 2.2 (Continued)

Herbs and spices	SD time (minute)	Maximum recovery of the desired chemical constituents in the essential oils after SD*	References
Pine (<i>Pinus ponderosa</i>)	5	α -pinene β -pinene	Zheljazkov <i>et al.</i> (2012b)
	10	Myrcene	
	160	δ -3-carene Limonene <i>cis</i> -ocimene α -terpinyl acetate germacrene-D α -murolene γ -cadinene δ -cadinene germacrene-D-4-ol	
Peppermint (<i>Mentha \times piperita</i> L.)	1.25	Eucalyptol	Cannon <i>et al.</i> (2013)
	20	Menthol Menthone Menthyl acetate	
	160	<i>t</i> -caryophyllene	
Lemongrass (<i>Cymbopogon flexuosus</i> Steud.)	40	Neral Geranial	Cannon <i>et al.</i> (2013)
	240	Caryophyllene oxide <i>t</i> -caryophyllene	
Palmarosa (<i>Cymbopogon martinii</i> Roxb.)	160	Geranyl acetate	Cannon <i>et al.</i> (2013)
Sweet sage wort (<i>Artemisia annua</i> L.)	2.5	α -pinene Camphene Eucalyptol Camphor Borneol	Zheljazkov <i>et al.</i> (2013a)
	10	<i>p</i> -cymene	
	20	β -chamigrene γ -himachalene	
	160	β -caryophyllene <i>trans</i> - β -farnesene Germacrene-D	
Anise (<i>Pimpinella anisum</i> L.)	5	Linalool Methyl chavicol <i>p</i> -anise-aldehyde <i>trans</i> -anethole	Zheljazkov <i>et al.</i> (2013c)
	480	<i>trans</i> -pseudoisoeugenyl-2-methyl Epoxy-pseudoisoeugenyl-2-methyl	

Table 2.2 (Continued)

Herbs and spices	SD time (minute)	Maximum recovery of the desired chemical constituents in the essential oils after SD*	References
Lavender (<i>Lavandula angustifolia</i> Mill.)	1.5	Cineole Fenchol	Zheljazkov <i>et al.</i> (2013d)
	15	Camphor	
	60	Linalool acetate	
Fennel (<i>Foeniculum vulgare</i> Mill.)	1.25	α -pinene β -pinene <i>p</i> -cymene Fenchone Camphor <i>cis</i> -anethole	(Zheljazkov) <i>et al.</i> (2013e)
	80	<i>trans</i> -anethole δ -3-carene γ -terpinene	
Chamomile (<i>Matricaria chamomilla</i> L.)	30	Farnesene	(Gawde) <i>et al.</i> (2014)
	60	Anethole	
	90	α -bisabolol oxide B	
	120	Chamazulene	
	180	Spathulenol	
	240	α -bisabolol oxide A	
Sage (<i>Salvia officinalis</i> L.)	360	Spiroether	(Zheljazkov) <i>et al.</i> (2014b)
	2.5	Camphor <i>cis</i> -thujene	
	10	α -pinene Camphene β -pinene Myrcene Limonene	
	160	β -caryophyllene α -humulene Verdifloral	

*Based on mean concentrations (%) of the total essential oil yield.

In another work performed by Mwaniki *et al.* (2015), EOs were extracted from chamomile flowers using the Clevenger distillation of multi-solvent solvent extraction method and by the standard Clevenger distillation. The authors tested numerous solvents including acetone, hexane, ethyl acetate, dichloromethane (DCM) and methanol. The efficiency of the extraction was determined according to the obtained final oil yield and the purity of the extract (as assessed by gas chromatography-mass spectrometry (GC-MS)). Results demonstrated that DCM as extracting solvent gave the highest yield of EOs, whereas hexane gave the lowest one.

Extraction systems using pressurised solvents with full automation have been introduced recently (ASE: automatised solvent extraction) (Luque de Castro & Priego-Capote, 2010) in order to provide effective and faster extraction, as well as favouring the use of lower solvent quantity. In this respect, numerous non-conventional technologies have been developed and evaluated for the extraction of EOs from herbs and spices; some of them are detailed in the next section of this chapter.

2.3 Novel Extraction Methods

2.3.1 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is one of the emerging, environmental-friendly and 'green' technologies. Nowadays, this technology is applied in analytical laboratories, so as to achieve fast and efficient extraction of EOs from plant matrices, and in industry, where valuable materials need to be extracted from plant sources under mild conditions. Generally, in SFE method, carbon dioxide (CO₂) is the ideal choice as a supercritical solvent due to the following reasons: CO₂ has a critical pressure of ≈ 73 bar and a critical temperature of $\approx 31^\circ\text{C}$, which are relatively low. In addition, CO₂ is non-flammable and a recyclable gas that is available at low cost and high purity, and can be separated from the extract by a simple depressurisation below the critical point (Koubaa *et al.*, 2015a). Moreover, the polarity of supercritical CO₂ (SC-CO₂) is similar to liquid pentane, which makes it suitable to be used as a green extraction method to obtain EOs from plant sources (Fornari *et al.*, 2012).

Up to now, several studies have evaluated the possibility of using SFE to extract EOs and optimised the extraction conditions. More recently, the composition of EOs and their anti-inflammatory activity extracted with SC-CO₂ from the brown seaweed *Undaria pinnatifida* was investigated (Kang *et al.*, 2016). A temperature of 45°C and a pressure of 20 MPa were found to be the optimal extraction conditions. The main compounds in the EOs extracted were palmitic acid, 2-(9,12-octadecadienyloxy)-(z,z)-ethanol, eicosa-5,8,11,14,17-pentaenoate and 5,7,11-eicosatrienoic acid, with the respective percentages of 26, 16, 13 and 12. Extracted EOs showed high anti-inflammatory activity. Singh *et al.* (2016) compared the SC-CO₂ extraction of EOs from the leaves of *Eucalyptus globulus* with other widely used methods (*e.g.*, solvent extraction (SE), hydrodistillation (HD) and ultrasound-assisted extraction (UAE)). The yields of EOs were of 2% (v/w) for HD, 2.2% for SE, 2.6% for UAE and 3.6% to SC-CO₂. Compared to other techniques, SC-CO₂ technique was found to be favourable to extract monoterpene, sesquiterpene and oxygenated sesquiterpene molecules. This work has demonstrated the importance of using the appropriate extraction method to obtain the high extraction yields.

Bogdanovic *et al.* (2016) examined the extraction of lemon balm (*Melissa officinalis*) by a two-step extraction method combining fractional subcritical and supercritical techniques. The yield of EOs fraction obtained at 10 MPa and 40°C was 0.45%, while the yields of the second fractions obtained at 30 MPa using different temperatures (*e.g.*, 25°C (higher pressure than critical pressure (HPCP-CO₂) but below the critical temperature), 40°C or 100°C (SC-CO₂)) was different (from 0.44% to 0.94%) depending on the applied extraction temperature. In another study, at the optimum extraction process parameters (21 MPa, 35°C , 3.77 g/min CO₂ flow and 160 min of extraction time), the

maximum yield of EOs (0.94%) was obtained from *Dracocephalum kotschyi* and this amount was higher than EOs extracted with HD method (0.73%) (Sodeifian *et al.*, 2016).

Uquiche and Garcés (2016) evaluated the effects of temperatures (40–60 °C) and pressures (9–15 MPa) on the recovery and antioxidant activity of the EOs from *Leptocarpha rivularis* leaves using SC-CO₂. Compared to hydrodistilled extract, the extraction yield (2.5 times) and antioxidant activity (5 times) of the supercritical extract at 40 °C and 15 MPa, were found to be higher. However, the extraction yield and antioxidant activity of the hydroethanolic extract were observed to be higher than SC-CO₂ extract. Similarly, Costa *et al.* (2015) evaluated the extraction yield, the compounds' profile, the antioxidant activity, and the organoleptic properties of the extracted volatiles from *Helichrysum italicum* subsp. *picardii* grown in Portugal using SC-CO₂ under different conditions (e.g., pressure (9 and 12 MPa)), and size of particles (<4 mm and 4 cm)). The application of an appropriate extraction technique, pressure and particle size were found to affect the extraction yields and chemical compositions of EOs. It was reported that the obtained SC-CO₂ extracts resembled to the scent of the plant growing in the field. Moreover, only the extract obtained by SC-CO₂ at 12 MPa and butylated hydroxytoluene (BHT) reduced the DPPH radicals.

Sage is an aromatic plant mainly used as an herbal tea. The extracts of this plant has been reported to play an important role in the treatment of different diseases (Arranz *et al.*, 2014; Eidi & Eidi, 2009). Recently, Akalin *et al.* (2015) evaluated the application of SC-CO₂ for extracting EOs from sage. The optimal extraction parameters to obtain the highest extraction yield were as follows: 280 °C temperature, 110 min extraction time and 11% (w/v) plant concentration in ethanol. The predicted oil yield obtained at the optimal conditions was 47.04 wt%, whereas the actual one was 49.21 wt%. The EOs were mainly composed of acid esters together with limonene, phenols, pyrroles and indoles.

Recently, Villanueva Bermejo *et al.* (2015) compared the extraction efficiency by using different 'green' solvents (e.g., limonene, ethanol and ethyl lactate) applied to pressurised liquid extraction (PLE) and SC-CO₂ at different conditions, in order to extract thymol from different thyme varieties. Although PLE was selected as the suitable technology for extracting thymol from thyme, SC-CO₂ technique allowed obtaining the highest concentrations of thymol. Saleem *et al.* (2015) evaluated the use of SC-CO₂ to extract EOs from cinnamon (*Cinnamomum zeylanicum*) bark. Compared to other conventional methods (HD and SD), more EOs yield was obtained using SC-CO₂. The most abundant compounds found in the EOs obtained by SC-CO₂ were limonene, cinnamaldehyde, naphthalene, copaene, heptane, bicyclo [4.2.0] octa-1,3,5-triene and 2-propenal.

Optimising the SC-CO₂ extraction of EOs and fatty acids from flixweed (*Descurainia sophia* L.) seeds using response surface methodology was recently conducted (Ara *et al.*, 2015). Results demonstrated that SC-CO₂ extraction method, under the optimised conditions, was more selective than the SD one. Methyl linoleate (18.2%), camphor (12.32%), *cis*-thujone (11.3%) and transcaryophyllene (9.17%) were found to be the most abundant components, when applying 355 bar pressure, 65 °C temperature, 150 µL methanol, 35 min dynamic extraction time and 10 min static extraction time. Oil yields achieved by SC-CO₂ extraction were ranging from 0.68 to 17.1% (w/w), while the one obtained using SD was only 0.25% (v/w). In another study, SC-CO₂ extraction conducted at different pressures up to 30 MPa was studied to define the dynamics of biologically active materials from a juniper (*Juniperus communis* var. *saxatilis*) (Aliev *et al.*, 2015). The SC-CO₂ extracts were reported to be more abundant by the compositions than EOs, and the

extract achieved at 10 MPa was compositionally similar to the EOs. SC-CO₂ extracts obtained at other pressures (e.g., 20 and 30 MPa) was different from the EOs and SC-CO₂ extract obtained at 10 MPa, with respect to the content of high molecular weight compounds.

Larkeche *et al.* (2015) investigated the extraction of EOs from *Juniperus communis* needles by SC-CO₂ operated at different processing conditions: pressure in the range of 10–30 MPa, the temperature within 308–328 K, solvent flow rate at 0.42 kg/h and an average particle diameter of 0.5 mm or no smaller than 0.315 mm. The maximum oil recovery yield was 6.55% and was obtained under 328 K temperature, 30 MPa pressure and <0.315 mm particle size. The most dominant extracted compounds were found to be germacrene, D- and L-octadecene. In another study, the effect of different supercritical fluid extraction parameters on the extraction of EOs from *Leptocarpha rivularisus-ing* was studied (Uquiche *et al.*, 2015). Extraction yield was positively influenced by temperature and negatively by pressure. The yield obtained with SC-CO₂ extraction (at 52 °C and 19.2 MPa) was reported to be higher (2.2 times) than those of EOs extracted with HD (2.11 g/100 g d.s.). Additionally, compared to the hydrodistilled extract, SC-CO₂ extracts displayed higher antioxidant activity.

Recently, non-polar and polar compounds from coriander (*Coriandrum sativum* L.) seeds (CS) were fractionated using SC-CO₂ extraction method (Zeković *et al.*, 2015). It was reported that SC-CO₂ had certain advantages compared to conventional methods in terms of higher extraction yield and better selectivity, as it provided the highest linalool content (877.07 mg/100 g CS) in the oil. A similar study was conducted by Pavlić *et al.* (2015) comparing the effect of SC-CO₂ and subcritical water extraction (SWE) methods to isolate EOs from coriander seeds (CS) with other methods (e.g., HD and solid-liquid extraction supplemented with methylene chloride). The highest extraction yields were achieved by Soxhlet (14.45%) and SC-CO₂ (8.88%) extraction methods (on 300 bar and 40 °C), while SWE at 100 °C led to the lowest yield of lipid extract (0.36%). Moreover, the highest extraction yields of γ -terpinene, (+)-limonene, linalool, camphor and geraniol (31.08, 23.98, 598.51, 26.64 and 19.54 mg/100 g CS, respectively) were obtained when SC-CO₂ was used as the extraction method.

2.3.2 Ultrasound-Assisted Extraction

Ultrasound technique has been commonly used over the last years in a laboratory scale to accelerate the extraction processes (Deng *et al.*, 2014; Koubaa *et al.*, 2015b), although the scale-up process of ultrasound-assisted extraction (UAE) is taking longer time to be widely accepted from food and pharmaceutical industries (Roselló-Soto *et al.*, 2015a). The basic principle of UAE to extract EOs from natural sources consists of generating sound waves (ultrasound frequency \approx 20 kHz), which create cavitation bubbles in the solution (Roselló-Soto *et al.*, 2015b), and produce enough energy to break the structures containing the oil in order to release it. Moreover, UAE can act as an emulsifier dispersing lipophilic molecules in water, thus facilitating the subsequent separation and purification of the EOs (Sereshti *et al.*, 2012).

2.3.2.1 Ultrasound-Assisted Solvent Extraction

There are several factors involved in the UAE process (e.g., power, frequency, time, temperature, type of solvent, pH of the solvent, liquid/solid ratio and solvent concentration)

(Roselló-Soto *et al.*, 2015a). The effects of UAE differ not only according to the plant matrix, but also the desired compound to be extracted. Therefore, there is a need to optimise UAE conditions for each compound and matrix individually.

For this reason, over the last two decades, several studies have been performed evaluating the feasibility of using UAE to recover EOs from different plant matrices. Due to the wide variety of the results and the large number of studies available, the focus in this chapter is only on the last five years. For instance, the effects of UAE (10, 20 and 45 min) on the extraction of EOs from fennel (*Foeniculum vulgare* Mill.) were investigated (Abbasian *et al.*, 2014). Before UAE, seeds were grinded and mixed with distilled water. The authors observed that compared to control (untreated sample), UAE enhanced the oil recovery. However, some compounds such as anethole, fenchone and α -pinene were significantly reduced.

Assami *et al.* (2015) investigated the impact of UAE+HD for the recovery of EOs from *Carum carvi* L. seeds. These authors found similar yields of volatile fraction ($\approx 1.7\%$) and a significant reduction in the extraction time (≈ 60 min) to obtain similar amounts of EOs ($\approx 80\%$) when they used UAE, compared to the untreated seeds.

Bernatoniene *et al.* (2016) studied the optimum UAE conditions to extract ursolic acid (UA), rosmarinic acid (RA) and oleanolic acid (OA) from rosemary leaves (*Rosmarinus officinalis* L.). The highest yields of UA, RA and OA were of 15.8 ± 0.2 mg/g, 15.4 ± 0.1 mg/g and 12.2 ± 0.1 mg/g, respectively.

Volatile compounds extraction from *Cannabis sativa* L. inflorescences assisted by UAE was also investigated and compared with traditional maceration process (Da Porto *et al.*, 2014). It was found that the extraction time could be significantly reduced when applying UAE compared to maceration. For instance, the authors observed that UAE treatments for a maximum of 5 min resulted in the same high yields of terpenes as maceration using significantly longer extraction times.

Smigielski *et al.* (2014) evaluated the impact of ultrasound-assisted maceration (20 min sonication time; 50 s impulsion time; 5 s pause time; 20°C temperature) on the yield of EOs from carrot seeds (*Daucus carota*). The authors obtained an improvement in EOs yield up to 33% under these conditions compared to the sample without UAE, being carotol ($\approx 35\%$), sabinene ($\approx 10\%$), α -pinene ($\approx 8\%$) and daucol ($\approx 4\%$) as the main constituents of the obtained EOs.

UAE (53 kHz, 32–52°C, 30–60 min) of EOs from clove (3–7% plant concentration) was optimised using response surface methodology (Tekin *et al.*, 2015). The authors observed that the predominant extracted compound was eugenol, followed by caryophyllene, and 2-methoxy-4-(2-propenyl) phenol acetate.

Furthermore, the potential of UAE to aromatise olive oil was evaluated by extracting EOs from *Carum carvi* L. seeds and mass transfer intensification of EO components from seeds to the bulk medium (Assami *et al.*, 2015). Carvone and limonene, which represented 99% of the caraway EOs were identified in olive oil, thus demonstrating the ability of UAE to aromatise olive oil as well as to enhance its quality and shelf-life. In addition, the flavouring rate after UAE was better than that obtained from the conventional method.

2.3.2.2 Combination of UAE with Other Techniques

UAE has also the potential to be combined with other conventional or novel technologies for improving the extraction of EOs. For instance, Pingret *et al.* (2013) evaluated

the potential of UAE to improve the conventional extraction of EOs from orange peels by HD process (Clevenger method). They found a significant decrease in the extraction time when they used UAE + Clevenger extraction compared to conventional Clevenger extraction used alone.

In another study, the impact of UAE and subsequent HD as a combined process to extract EOs from *Elettaria cardamomum* L. seeds was evaluated (Morsy, 2015). The ultrasonic power and sonication time were optimised. The authors found a significant reduction of extraction time and improved extraction efficiencies of 1,8-cineole (26.59%–39.34%) and terpinyl acetate (22.94%–40.56%) when UAE was combined with HD compared to the conventional processes. The application of UAE involved shortening the extraction time, enhancing the extraction yield and producing high extract quality.

UAE can be combined with pressurised fluids, too. In this respect, UAE was combined with supercritical CO₂ to improve the extraction of EOs from masson pine (Yu *et al.*, 2012). Under the optimum conditions (UAE (22 KHz, 250 W) + SC-CO₂ (18 MPa, 60 °C, 90 min, a CO₂ flow rate of 12 kg/h)), the extraction yield for EOs was approximately 88%. Longifolene (44%) was the predominant compound found in the EOs, followed by α -pinene (28%) and caryophyllene (6%).

More recently, UAE was applied in combination with subcritical fluid extraction in a process called ultrasound-enhanced subcritical water extraction (USWE) to extract EOs from *Kaempferia galangal* L., a plant belonging to the family of *Zingiberaceae* (Ma *et al.*, 2015). The authors optimised the processing conditions and compared the results of the optimised process with subcritical water extraction (alone) and SD extraction. Under the optimum conditions (UAE (20 kHz, 250 W/L) + subcritical water extraction (10 MPa, 120 °C, 20 min)), the authors extracted 25.80 mg/g of ethyl *trans-p*-methoxycinnamate, which was significantly higher than SWE (alone) and SD extraction.

In addition, the combination of UAE + ultrasound assisted emulsification microextraction (USAEME) was used to pre-concentrate EOs from *Elettaria cardamomum* Maton (Sereshti *et al.*, 2012). When this technique was used under the optimum conditions (32.5 °C, 10.5 min, 120 ml extraction solvent (C₂H₄Cl₂)), the major extracted compounds in EOs were terpenyl acetate (46.0%), 1,8-cineole (27.7%), linalool (5.3%), terpineol (4.0%) and linalyl acetate (3.5%).

The combination of UAE with dispersive liquid-liquid microextraction (UAE-DLLME) also showed promising effect to extract and pre-concentrate the EOs from *Edgeworthia chrysantha* Lindl flowers (Wen *et al.*, 2014). Under the optimum conditions (UAE (10 min) + DLLME (toluene as the extraction solvent and acetone as the dispersive solvent)), 36 compounds were identified in the EOs.

Other authors developed an innovative technique known as ultrasound-microwave hybrid-assisted extraction coupled with headspace solid-phase microextraction (UMHE-HS-SPME) to isolate and determine EOs of dry roots from *Angelica dahurica*, which are commonly used in traditional Chinese medicine (Feng *et al.*, 2014). In brief, the authors combined UAE with MW-assisted extraction (MAE) and coupled with headspace solid-phase microextraction. EOs were isolated, extracted and concentrated in one step and the impact of different extraction conditions (coating with fibres, power of ultrasound and time of irradiation) were evaluated, optimised and compared to those obtained with conventional SD. It was found that UMHE-HS-SPME allowed isolating and identifying more compounds in the EOs compared to SD. In addition, this new

method has the capacity to extract oxygenated and higher boiling point compounds from *A. dahurica*, as well as reducing the extraction time (10 min (UMHE-HS-SPME) versus 6 h (SD)) and organic solvent consumption (no organic solvent) compared to SD.

2.3.3 Ohmic-Assisted Hydrodistillation

Ohmic heating is an innovative method of heat treatment. During this treatment, a plant material constitutes an electrical resistor, which allows the dissipation of electrical energy as heat when electricity passes through it. This phenomenon leads to increase the temperature of the medium in a rapid and uniform way. Ohmic heating could be used in different applications including extraction, evaporation, blanching, drying and fermentation. Ohmic-assisted extraction is a sophisticated process, which can provide faster extraction kinetics at lower cost. Indeed, the extraction time can be reduced by a factor of two compared to conventional extraction (Gavahian *et al.*, 2012). Conventional thermal treatment consists of producing heat by conduction, which involves slow heating rate and causes a gradient of temperature in the materials. These differences in the temperature decrease the extraction efficiency and damage the quality of the product. However, the material having undergone ohmic heating may be heated rapidly and in uniform way throughout the bulk of a material without affecting the material quality (Pathak, 2011; Rostagno & Prado, 2013; Sun, 2014).

Ohmic-heating technique has been applied to extract EOs from herbs and spices. For instance, the impact of ohmic-heating HD on the extraction of EOs from *Prangos ferulacea* Lindle was compared to conventional HD (Seidi Damyeh & Niakousari, 2016). Results showed that ohmic-heating HD provided similar yields than HD, however, the processing time as well as the energy consumption were reduced to the half, when using this novel technology. Moreover, the extracted EOs, as analysed by GC-MS, showed significant differences in the amount of the main components between HD and ohmic-heating HD. Selected components such as limonene and (E)- β -ocimene were better extracted by ohmic-heating HD, yielding 13.32% and 23.60%, respectively. The authors associated these findings with the occurrence of internal heating and electroporation taking place during ohmic heating, and hence concluded the potential of this extraction method as 'novel green technology'.

In another recent work, EOs were extracted from *Mentha piperita* using ohmic-assisted HD (Gavahian *et al.*, 2015b). Different frequencies (25, 50 and 100 Hz) and ohmic-heating HD intensities (220 and 380 V) were tested and the obtained results were compared to HD. Results showed a significant reduction in the processing time between conventional HD (1 h) and ohmic-heating HD (13.54 min and 19.71 min, when processing at 220 V and 380 V, respectively). The same research group investigated the impact of ohmic-heating HD for the extraction of EOs from *Thymus vulgaris* L. and compared the results with conventional HD (Gavahian *et al.*, 2012). The authors reported significant decrease in the extraction time, from 1 h (for HD) to 24.75 min (for ohmic-heating HD). Moreover, they reported significant differences in the composition of EOs extracted by both techniques. In another study, they compared ohmic-assisted HD to microwave assisted extraction for the recovery of EOs from *Mentha piperita* (Gavahian *et al.*, 2015a). Results were in agreement with other investigations in which the overall processing time can be shortened from 1 h to less than half an hour compared to HD. In terms of energy usage, the lowest consumption was reported for

ohmic-heating HD. All of these studies demonstrated the great interest in replacing conventional HD by ohmic-heating HD, as well as its potential as novel and 'green' extraction technique.

2.3.4 Pulsed Electrical-Assisted Extraction

Interest in the application of non-thermal processing techniques to treat food and plant materials has been increased in the last two decades. Pulsed electric field (PEF) is one of the most appealing technologies amongst the non-thermal techniques, which is currently used in processing of various foods around the world. The application of PEF treatment is based on the effect of electroporation on cells, which induces local structural alterations and breakdown at the cellular membranes. Permeabilisation of cell membranes by electroporation is a promising technique caused by applying external short and intense electric pulses. The impact of PEF on a cell can be defined as follows: (1) transmembrane potential formation in the cytoplasmic membrane, (2) pores formation and (3) changes in the cell membrane pore size and number (Figure 2.2) (Roohinejad, Everett, & Oey, 2014). Electroporation can be reversible or irreversible, depending on the field intensity applied (Puértolas *et al.*, 2016). In reversible electroporation, the applied electric field is close to the cell critical value giving thus to the membranes the possibility to return back to their original shape and the recovery of their integrity. However, in irreversible electroporation, by applying electric field strengths higher than the critical value, loss of cell viability occurred, permanent holes will be formed in the

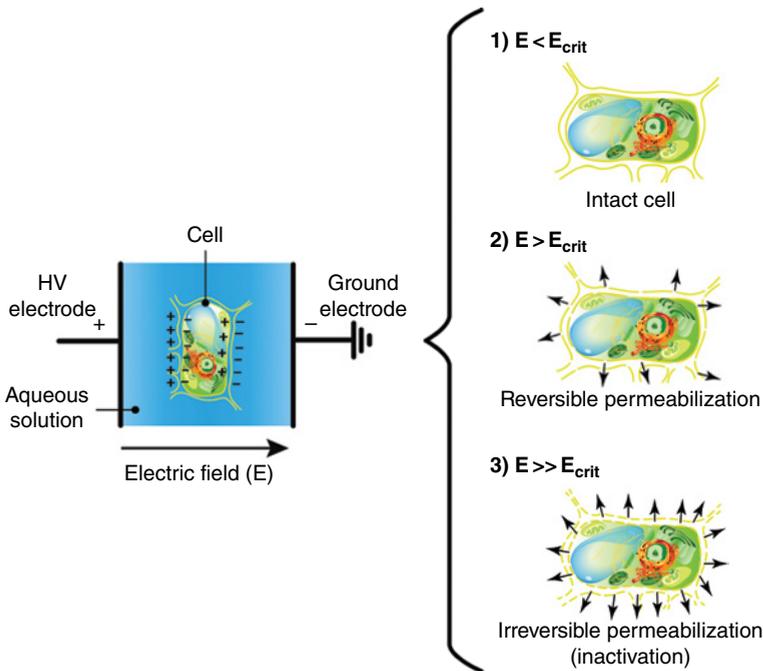


Figure 2.2 Schematic mechanism of membrane permeabilisation induced by an external electrical field. Irreversible, large membrane pores are formed by increasing treatment intensity.

cell membrane and consequently the release of intracellular components or entry of extracellular materials is possible. Usually, reversible electroporation is needed for the biotechnological applications of PEF, while to inactivate microorganisms and to improve the mass transfer; irreversible electroporation is required (Toepfl, Heinz, & Knorr, 2005).

Many studies have shown the feasibility of using PEF treatment to improve the extraction of food active compounds from plant sources. For instance, the application of PEF processing was demonstrated to enhance the extraction of different intracellular compounds such as phenolics and anthocyanins from fruits and vegetables (Barba *et al.*, 2015; Leong *et al.*, 2016; López-Giral *et al.*, 2015), sugar from sugar beet (El Belghiti & Vorobiev, 2004; Loginova *et al.*, 2011b; López *et al.*, 2009), betalains from red prickly pear and red beets (Koubaa *et al.*, 2016a; Loginova *et al.*, 2011a) and carotenoids from carrot purée (Roohinejad *et al.*, 2014). Moreover, numerous works have investigated the application of PEF to extract oil from olives (Abenoza *et al.*, 2013; Puértolas & Martínez de Marañón, 2015), sesame seeds (Sarkis *et al.*, 2015), rapeseeds (Guderjan *et al.*, 2007) and sunflower seeds (Shorstkii *et al.*, 2015). However, up to now, only few studies were conducted to evaluate the effectiveness of PEF processing for improving the extraction of EOs from plant sources.

The cultivation of white rose (*Rosa alba* L.) for the preparation of white oil-bearing rose is important for the Bulgarian rose industry due to the high resistance of this plant against low temperatures and susceptibility to different plant diseases. However, the EOs content of this plant is low (0.015–0.030%) and results in a low oil yield and an expensive final product. Recently, Dobрева *et al.* (2010) evaluated the feasibility of using PEF processing for improving the yield and quality of EOs during distillation of white rose. PEF pre-treatment with an energy input of 10 kJ/kg and 20 kJ/kg resulted in an increase of 13–33% in the EOs yield. Moreover, the application of PEF facilitated the diffusion processes in the blossom tissues and reduced the distillation time from 2.5 to 1.5 h compared to the conventional extraction method. The determination of oil yield, chemical composition and olfactory evaluation revealed that the optimal yield and quality could be achieved using PEF pre-treatment at energy input of 10 kJ/kg followed by 1.5 h distillation time. At these optimal conditions, the yield was increased up to 24% and no undesired changes were observed on the olfactory properties. Thus, it is concluded that the application of PEF pre-treatment in an industrial scale can be used to enhance the production capacity and avoid unfavourable changes in the rose oil composition due to fermentation (Dobрева *et al.*, 2010).

Another study has been conducted to evaluate the effect of PEF on the yield and chemical composition of rose oil (*Rosa damascena* Mill.) (Tintchev *et al.*, 2012). Application of PEF processing with an energy input of 10 kJ/kg and 20 kJ/kg enhanced the oil yields (2–46%) and reduced the distillation time. Moreover, an increase of terpene alcohols/hydrocarbons ratio from 33% to 285% was found in the treated rose oil. Application of PEF pre-treatment facilitated the diffusion processes in the blossom tissues and reduced the distillation time from 2.5 to 1.5 h compared to the conventional processing technique. The optimal PEF pre-treatment conditions were found to be an energy input of 10 kJ/kg and distillation time of 1.5 h. At these optimal conditions, a yield increase of 46% was observed; the EOs have a balanced composition and rose oils with odor close to the standard rose oils available on the market were obtained.

Recently, another study was carried out to improve the distillation process of different EO crops such as fresh herb *Nepeta transcaucasica* Grosh, dry seeds of *Coriandrum*

sativum L., and *Levisticum officinale* Koch using PEF treatment (Dobrevia *et al.*, 2013). It was found that the effect of PEF treatment with an energy input of 20 kJ/kg followed by distillation highly depends on the EO crops investigated. The oil yield was decreased by 20% in herbal *Nepeta transcaucasica* Grosh and by 5% in *Coriandrum sativum* L. seed. However, the application of PEF treatment enhanced the oil yield by 67% in the seeds of *Levisticum officinale* Koch. Moreover, the quality and the composition of the treated EOs were not affected using PEF treatment.

Patchouli (*Pogostemon cablin*, Benth) is a native plant of tropical Asia and it is mainly grown for its valuable EOs (Santos *et al.*, 2011; Sugimura *et al.*, 1995). Patchouli EO is well known for its medicinal and aromatic properties and it is used in the food and pharmaceutical industries (Ramya *et al.*, 2013). However, in patchouli leaves, the EOs are stored in special cells called glandular trichome (GT), which makes their extraction difficult (Maeda & Miyake, 1997). Recently, the effect of PEF treatment on damaging the cell wall of GT of patchouli leaves and the quality of patchouli EOs were studied (Soeparman *et al.*, 2013). Patchouli leaves were treated with PEF at electric field strengths between 50–150 V/cm for 2–3 s. The results showed that PEF treatment with electric field strengths of 100 V/cm at 2 s damaged more than 95% of the GT cell walls and increased the oil yield by 35%.

2.3.5 Microwave-Assisted Extraction

The use of microwave (MW) and dielectric heating dated back to the late 1970s (Koubaa *et al.*, 2016b). The efficient volumetric heating provided by MW makes this technology much more efficient than other thermal treatments (Chemat & Cravotto, 2013; Ramanadhan, 2005; Singh & Heldman, 2001). The frequency of the electromagnetic energy generated by MW equipment is in the range of 0.3–300 GHz (Barba *et al.*, 2016). This energy is then transferred to heat following dipole rotation and ionic conduction (Jain *et al.*, 2009) (Figure 2.3A). The principle is based on the direct impact on polar compounds composing the extracting solvent or the matrix being treated. Due to its numerous beneficial properties (achieving high rates of heating, reducing the treatment time, obtaining better heating homogeneity, and requiring low maintenance, with safe handling) compared to the conventional extraction methods, MW technology has been applied for decades in the field of food processing for pasteurisation and sterilisation (Ahmed & Ramaswamy, 2007), thawing (Li & Sun, 2002), drying (Zhang *et al.*, 2006) and EOs extraction (Cardoso-Ugarte *et al.*, 2013). Applying MW for the extraction of EOs from herbs and spices may lead to minimal changes in the flavours compared to conventional heating. Amongst MW extraction methods, vacuum MW hydrodistillation (VMHD), MW hydrodiffusion and gravity (MHG) and solvent free microwave extraction (SFME) (Figure 2.3D) were the most applied.

2.3.5.1 Vacuum Microwave Hydrodistillation (VMHD)

VMHD was first patented by Archimex company for the extraction of EOs from plant materials (Mengal *et al.*, 1993). The method is performed in two stages: (1) heating the plant material with MW (the matrix is humidified if used in dry state), and (2) creating vacuum (0.1–0.2 bar) in the system, which allows the azeotropic distillation of the water-EOs mixture (Poux *et al.*, 2015) (Figure 2.3B). The operating temperature is generally lower than 100°C, which avoids the degradation of the most thermo-sensitive

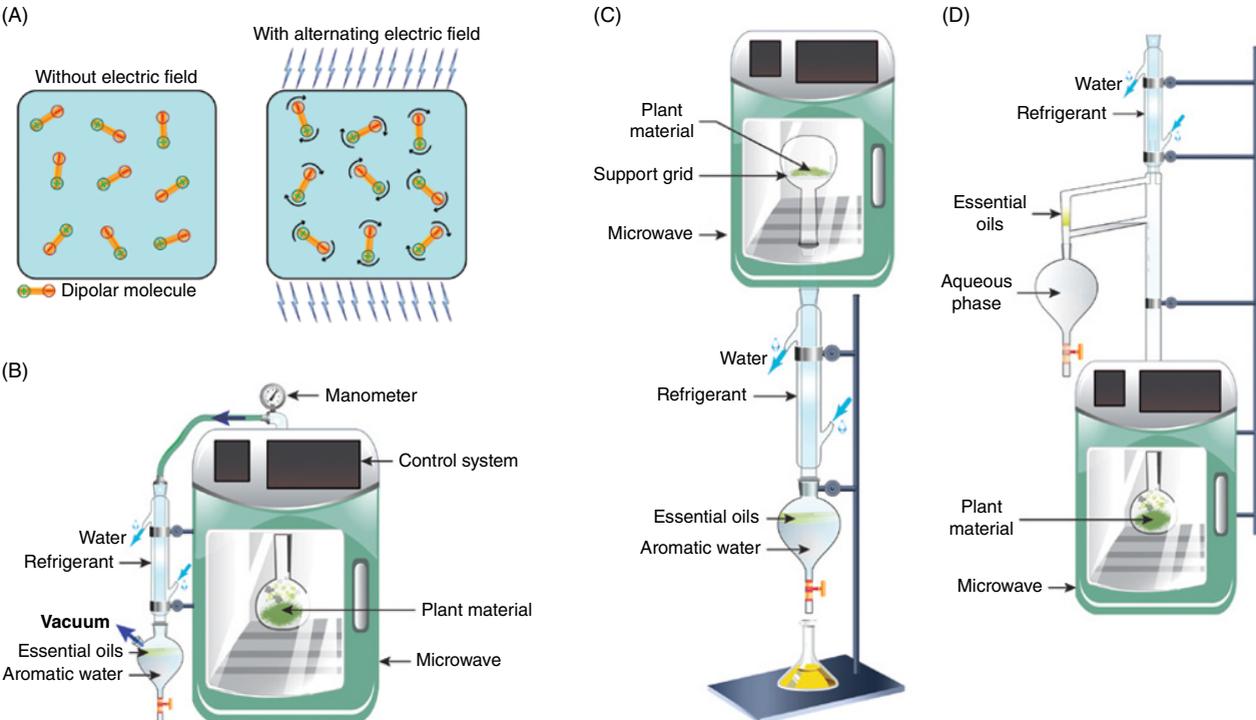


Figure 2.3 Microwave experimental set-up used at laboratory scale for the extraction of EOs from herbs and spices. A. Influence of electromagnetic field on dipolar molecules. B. Vacuum microwave hydrodistillation. C. Microwave hydrodiffusion and gravity. D. Solvent-free microwave extraction.

compounds. In addition, the technique allows reducing the operating time by a factor of 5–10 compared to HD (Li *et al.*, 2014), as well as reducing the power consumption. It has been reported that the recommended incident power is ranging from 0.2 to 1 kW/kg of raw material (Poux *et al.*, 2015).

2.3.5.2 Microwave Hydrodiffusion and Gravity (MHG)

MHG (Figure 2.3C) is another green technique proposed in 2008 (Vian *et al.*, 2008), which could be used for the extraction of EOs from plant matrices. It is based on MW heating and earth gravity at atmospheric pressure, and designed for both laboratory and industrial applications. Plant material is first placed inside the MW equipment, without additional water. Due to the internal heating by MW, EOs are released *in situ* within the water constituting the matrix. This phenomenon is called hydrodiffusion, which allows the diffusion of the extract (EOs + water) outside the cells and its recovery by gravity. A cooling system allows obtaining liquid extracts composed of two phases (EOs (light phase) in the top, and aromatic water (heavy phase) in the bottom), which later could be separated using a 'separatory funnel' or 'Florentine flask'. The novelty of the method is that it allows the extraction of EOs without distillation and evaporation, which is very useful to save the energy being used for heating (Zill-E-Huma, 2010).

For instance, MHG was evaluated to extract EOs from two aromatic herbs: spearmint (*Mentha spicata* L.) and pennyroyal (*Mentha pulegium* L.) (Vian *et al.*, 2008). The extraction was performed at atmospheric pressure without additional solvents, and was compared to HD. The obtained results demonstrated that EOs could be extracted in only 15 min using MHG achieving similar yields and composition compared to HD after 90 min of extraction. Furthermore, MHG allowed saving up to 90% energy consumption compared to HD.

The same research group investigated the extraction of EOs from rosemary (*Rosmarinus officinalis* L.) leaves by means of HD and MHG (Bousbia *et al.*, 2009). Results showed that MHG method shortened the extraction time from 3 h using HD, to 15 min using MHG. In addition, the authors reported that MHG technique has numerous advantages compared to HD, including lower energy consumption, cleaner features due to the absence of organic solvents or water and increased biological (antibacterial and antioxidant) activities, due to the higher amount of oxygenated compounds. By analysing the tissue structure using scanning electron microscopy, important changes such as clearly broken and damaged cells have been observed for the material treated with MHG, compared to that treated with HD.

In a similar study, EOs were extracted from dried caraway seeds using MHG, and results were compared to conventional HD (Farhat *et al.*, 2010). For the same yield and composition, MHG is more efficient in reducing the extraction time from 300 min to 45 min, thus saving the energy required for heating and producing better, pure and natural product.

More recently, EOs were extracted from four different *Lamiaceae* plants (*Salvia officinalis* L., *Lavandula angustifolia*, *Origanum vulgare* L. and *Ocimum basilicum* L.) using MHG and HD (Binello *et al.*, 2014). The authors reported that both methods allowed obtaining excellent results; especially the EOs obtained under MHG were very similar to those achieved with HD. However, using MHG resulted in significant difference in the physicochemical properties of the obtained EOs due to the variation in the compounds' polarity.

Similarly, EOs were extracted from lavandin flowers using conventional (*e.g.*, hydro-distillation (HD), steam distillation (SD)) and non-conventional (ultrasound- and MW-assisted extraction) techniques (Périno-Issartier *et al.*, 2013). Although non-significant differences were observed in the yield and profile of EOs obtained by both techniques, MHG was revealed to be the most efficient due to shortening the extraction time from 220 min (using HD) to only 30 min without altering the sensory properties.

2.3.5.3 Solvent-Free Microwave Extraction (SFME)

SFME was first developed by Chemat's team in 2004 (Lucchesi *et al.*, 2004a, 2004b). The method consists of extracting EOs from plant matrices without adding water or any other solvent. SFME is also called MW-assisted dry distillation, as it is neither a modified MAE, which involved the use of organic solvents, nor a modified HD, which uses large amount of water (Filly *et al.*, 2014) (Figure 2.3D). The principle is the same as previously described for MHD. However, here, the evaporated water is refluxed to the extractor maintaining the same amount of water in the plant material. SFME was evaluated for numerous herbs and spices and the published works are summarised below.

The extraction of EOs from oregano, a perennial and shrubby herb, was investigated (Bayramoglu *et al.*, 2008). The impact of MW power and time on the extraction efficiency (yield and composition) was investigated. The authors were also interested in examining the specific gravity, refractive index and solubility in alcohol of the extracted compounds. Results showed that the yield of EOs extracted by SFME was higher than that obtained by HD, with 0.054 ml/g and 0.048 ml/g, respectively. In addition to the highest extraction yield, SFME consumed less energy (saving up to 80%) than HD, when applying at a MW power of 622 W. The composition of EOs was assessed using GC-MS, revealing the presence of thymol as the major aroma compound (650–750 mg/ml) in an oregano extract. By comparing both compositions of the EOs obtained by SFME and HD, no significant differences were observed.

In another work, SFME technique was applied for the extraction of EOs from three aromatic herbs: basil (*Ocimum basilicum* L.), garden mint (*Mentha crispa* L.) and thyme (*Thymus vulgaris* L.), and the obtained results were compared to HD (Lucchesi *et al.*, 2004a). The authors reported that the extraction time could be reduced from 4.5 h to 30 min, using HD and SFME, respectively, while achieving oils with similar compositions and yields. Although EOs produced by HD and SFME have similar aromatic profiles, higher amounts of valuable oxygenated compounds were obtained using SFME. Moreover, this technique is energy saving, and less plant material is needed for the extraction.

The same research group investigated the extraction of EOs using SFME and HD from three spices: ajowan (*Carum ajowan*, Apiaceae), cumin (*Cuminum cyminum*, Umbelliferae) star anise (*Illicium anisatum*, Illiciaceae) (Lucchesi *et al.*, 2004b). In addition to avoiding the use of solvents when applying SFME, the obtained results demonstrated reduced extraction times (from 8 h to 1 h) compared to HD. Moreover, EOs obtained by SFME could be directly analysed by GC-MS without additional steps of cleaning or solvent exchange.

SFME was also applied for the extraction of EOs from *Elletaria cardamomum* L., (Lucchesi *et al.*, 2007) (commonly known as green or true cardamom), which is a herbaceous perennial plant belonging to ginger family. The authors compared the results

obtained by SFME to those obtained by conventional HD. The analysis of the EO compositions by GC-MS, obtained by both methods, showed that EOs obtained by SFME were dominated by the presence of oxygenated fraction, which is composed of odoriferous aromatic compounds highly sought for better fragrance. In addition, the efficiency of the extraction was investigated using scanning electron microscopy. Results demonstrated advanced destruction of the seed tissues treated by SFME, as compared to that treated by HD.

In another study, SFME method was also applied for the extraction of EOs from rosemary (*Rosmarinus officinalis* L.) leaves, and the results were compared to conventional HD (Okoh *et al.*, 2010). Analysis of the composition of EOs by GC-MS showed comparable profiles with 24 and 21 compounds identified in HD and SFME EOs, respectively. The extraction yield of EOs using SFME was relatively higher than that obtained by HD, with 0.39% and 0.31%, respectively. Moreover, the amount of oxygenated monoterpenes (*i.e.*, borneol, camphor, terpene-4-ol, linalool, α -terpeneol) was higher in SFME extract, compared to HD extract, with 28.6% and 26.98%, respectively. In contrast, the authors reported more monoterpene compounds (*i.e.*, α -phellanderene, α -pinene, β -pinene, γ -terpenene, camphene, myrcene, 1,8-cineole, *trans*- β -ocimene and *cis*-sabinene hydrate) in HD EOs, compared to SFME extract, with 32.95% versus 25.77%, respectively.

2.4 Conclusions

Numerous methods have been applied for the extraction of EOs from herbs and spices. Although HD and SD are the most applied methods, several non-conventional technologies have been evaluated, especially in respect to EO recovery, and a great potential has been attributed to these non-conventional methods. For instance, ultrasound, microwave, supercritical fluid, pulsed electric fields and ohmic-heating techniques applied alone or combined with other techniques are potent tools to obtain EOs in a 'green' way from natural resources such as aromatic plants, spices, fruits and flowers in shorter times and with high extraction efficiency as with conventional processes. Moreover, these techniques have the potential to reduce the energy cost and wastewater and perform in some cases even a solvent-free extraction. Electroporation, for example, by PEF treatment, can be used to disrupt the cell walls and improve the extractability of EOs from plant materials. Although several studies evaluated the application of PEF processing on improving the extractability of hydrophobic and hydrophilic bioactive compounds as well as plant oils, very limited studies have been conducted on improving the extraction of EOs. Thus, further studies need to be carried out to address these issues.

Acknowledgements

Leong acknowledged the research grant from Deutscher Akademischer Austauschdienst. Shahin Roohinejad would like to acknowledge the Alexander von Humboldt Foundation for his postdoctoral research fellowship award.

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3

Identification of Essential Oil Components

Elena E. Stashenko and Jairo Rene Martinez

Research Centre for Biomolecules, CIBIMOL-CENIVAM, Universidad Industrial de Santander, Bucaramanga, Colombia

3.1 Introduction

Essential oil (EO) commercial value and, above all, its application in different branches of industry, are directly related to its physicochemical properties, odour, biological activity and, primarily, to its chemical composition. This involves not only the most abundant compounds, but also those present at trace levels. The complete physical, chemical and sensory characterisation of the oil is mandatory. The oil characterisation process is divided into several steps, each of which may be of lower or higher relevance to decision making, depending on the EO application. For example, if an EO is predominantly composed of monoterpene or sesquiterpene hydrocarbons, not soluble in water-alcohol, then its use will be less attractive to the perfume industry; safrole-rich oils, have a restriction for use in food products, for example, beverages due to its potential genotoxic and carcinogenic effects (SCE, 2002); while in contrast, oils rich in thymol and carvacrol (antiseptic compounds), would be of great interest for incorporation into herbal medicines, cosmetics or toiletries and antimicrobial protection agents (stabilisers, antioxidants). The requirements and importance of each stage of the EO characterisation may vary. Information on cytotoxicity, genotoxicity, allergenicity and other biological properties, may be very important, if the EO is used in herbal medicine and foodstuffs. For example, the use of EOs containing estragole is restricted because this substance is considered a potential carcinogenic (McDonald, 1999). The EO fragrance and sensory evaluation as well as its solubility in water-alcohol mixture will be important for its use in perfumes, lotions, creams or as a flavouring ingredient in cleaning products; non-irritability, not allergenicity and the absence of photosensitising effects of an EO component are requisites for use in cosmetics or personal care products, amongst others (Berger, 2007).

The complete characterisation of an EO is achieved when the following steps are performed:

- 1) Sensory evaluation. It is made by a panel of experts and tasters, called 'noses'. It is decisive in selecting the scented oil as an ingredient, as a fragrant note in perfumes or related products.

- 2) Determination of physicochemical properties. The overall EO appearance, that is, texture, colour and fluidity, are described and physicochemical parameters are determined in accordance to various international standards (ISO, International Organisation for Standardisation). There are standardised procedures for the determination of density, refractive index, solubility in alcohol, optical rotation, peroxide index, ester number, acid number, freezing point, ignition (flash point) and other parameters (UNIDO & FAO, 2005). The physicochemical parameters must be compiled in the technical data report, which accompanies the product when it is marketed.
- 3) Determination of biological properties. These studies are important for those oils used in consumer products. In flavouring, for example, the antioxidant and antimicrobial activities should be proven, along with the organoleptic, sensory evaluation and stability tests, to contribute to determine if the EO serves as natural ingredient in consumer products. All this, provided that no photosensitising or allergenic substances appear in its composition (e.g., furanocoumarins, psoralen).
- 4) Determination of the chemical composition. It is one of the most important analyses performed on an EO. The technical report, together with the physicochemical parameters, must contain the respective chromatogram and chemical composition table, with names of constituents and their relative amounts (%). The main constituents should be quantified using standard compounds. The set of physicochemical properties and chemical composition of an EO distinguishes it and constitute the basis for the assessment of purity, price and applicability of this product in any major market (agricultural business, food, pharmaceutical, perfumes, fragrances and toiletries industries) (de Silva, 1995).

Gas chromatography (GC) is the basic analytical technique used to determine EO composition. GC is used intensively in many branches of science. Chromatography has its greatest strength in separating mixture components. A large variety of detection systems can generate an electric signal in response to the components of the fluid that exits the chromatographic column that are different from the carrier gas. This signal and its time dependence are used to determine the number of components and their proportion. However, additional information is required to establish the chemical structure of these separated and quantified compounds. This information is provided by detectors whose signal is related to the chemical structure, which are spectroscopic or spectrometric detectors (Jennings & Shibamoto, 1980). Amongst them, the most used, is the mass spectrometric detector (MS). The signals from this detector, the molecule's mass spectrum, are closely related to its chemical structure and inform on molecular weight, functional groups present and, in certain circumstances, geometry and spatial isomerism of the molecule. The elemental composition of the substance can be established when high-resolution mass spectrometry is employed. The combination of GC and MS results in a very powerful tool for EO chemical characterisation. Despite the vast analytical strength and reliability which gas chromatography-mass spectrometry (GC-MS) enjoys today, it is important to clearly discern its scope, limitations and drawbacks. The main limitations are set by the type of sample (molecular weight, volatility, polarity and thermostability of its components). Two fundamental problems stand out: (1) co-elution of components in the chromatographic column and (2) unique (non-ambiguous) identification of the components (the same retention times, or different

retention times but similar mass spectra, or both). The following sections of this chapter describe various procedures and techniques already available or under development to address these difficulties for the case of EO analysis.

3.2 Essential Oils as Multicomponent Complex Mixtures

Essential oils are complex fragrant volatile liquids, composed of plant secondary metabolites isolated by distillation of aromatic species or by cold-pressing of citrus fruits. They are mixtures which may contain more than 300 substances (Sandra & Bicchi, 1987). Hydrocarbons, ethers, alcohols, aldehydes, ketones, carboxylic acids and esters are the functional groups most frequently found in the EO constituents. Most of the EO components belong to the terpenoid families (C_{10} and C_{15}). However, phenyl propanoids and other phenolic derivatives, are the main constituents of several EO. The concentration of the constituents in EOs can vary over several orders of magnitude, from $\mu\text{g}/\text{kg}$ to g/kg . Many EO components have isomers, for example, possess positional, geometric, spatial and optical isomerism. All this large diversity of chemical features and relative abundances pose two main problems to reach the analytical goals, namely: (1) the thorough separation of all the constituents, and (2) their unambiguous structural determination.

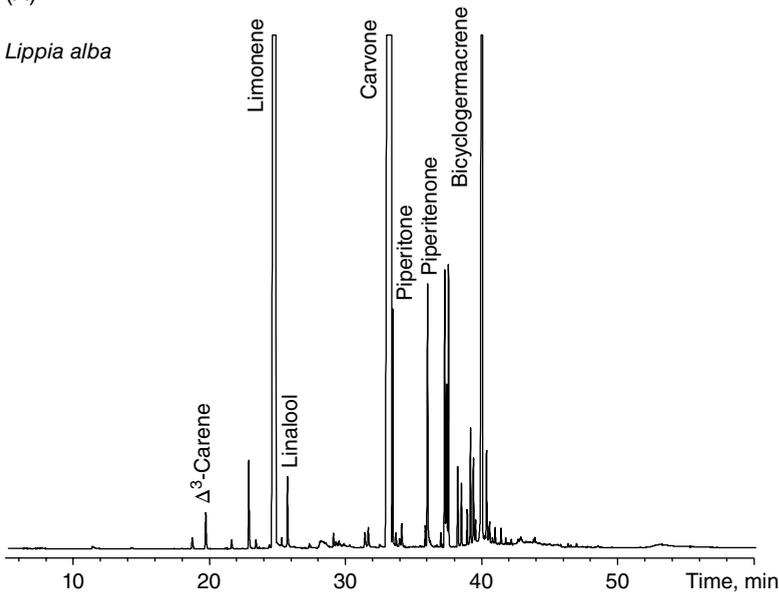
3.2.1 Classification and Main Components

EOs consist mainly of one or more of the following classes of volatile substances, namely: (1) Hydrocarbon terpenes (C_{10} , C_{15}) and their oxygenated derivatives (terpenoids); (2) Phenolic compounds and their derivatives; (3) Other oxygenated molecules (alcohols, ethers, acids, *etc.*); (4) Phenyl propanoids; (5) Heterocyclic compounds, usually containing nitrogen or sulfur atoms. All these substances are of relatively low-molecular-weight ($<300\text{Da}$), nonpolar or moderately polar, are volatile and each one has its characteristic odour. Terpenoids (C_{10} , C_{15}) are the largest group of compounds present in EOs.

Chemically, the terpenes result from binding several molecules (units) of isoprene, an unsaturated hydrocarbon with condensed formula C_5H_8 . Per the number of fused isoprene units, terpene groups are distinguished as monoterpenes ($n=2$, $C_{10}H_{16}$) and sesquiterpenes ($n=3$, $C_{15}H_{24}$). Isoprenes can be coupled together in multiple ways and form numerous isomers. In this family of compounds (terpenes) all kinds of isomerism, for example, geometrical, optical and stereoisomerism, can occur. These are the most common members of EOs, together with their numerous oxygenated derivatives, including aldehydes, ketones, alcohols and esters. Hydrocarbon terpenes (C_{10} , C_{15}) do not mix well with water, but their oxygenated derivatives (alcohols, ketones, ethers or esters) themselves are more soluble in water or in hydroethanolic solutions (Baser & Buchbauer, 2009).

EOs can be classified into three main groups according to the family of chemical compounds that prevail in the mixture: (1) Monoterpenoid type: most of the substances present in the oil are monoterpenes and their oxygenated analogs (*e.g.*, pine, rosemary or mint oils); (2) Sesquiterpenoid type: when sesquiterpenes and their oxygenated

(A)

Lippia alba

(B)

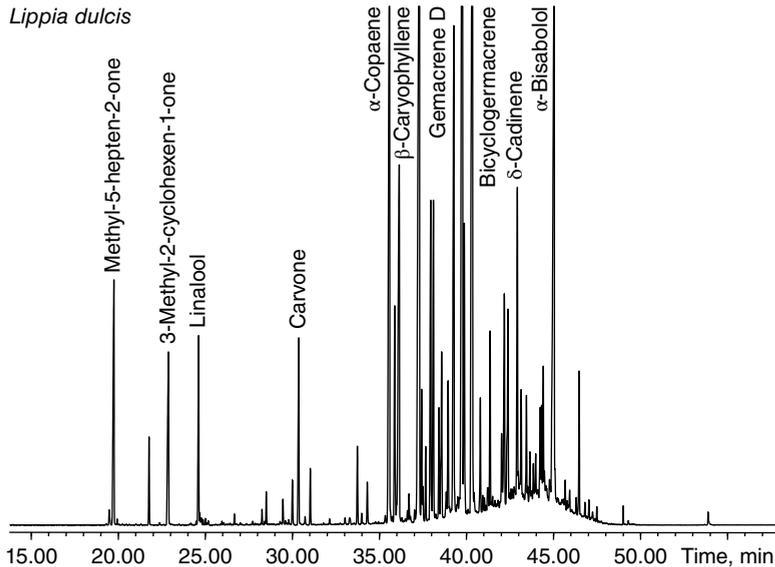
Lippia dulcis

Figure 3.1 Chromatograms obtained by GC-FID and the major compounds of the essential oils isolated by hydrodistillation from three aromatic plants of *Lippia* genus (Verbenaceae family). **A.** *Lippia alba* (Monoterpenoid type); **B.** *Lippia dulcis* (Sesquiterpenoid type) and **C.** *Lippia micromera* (Phenolic type).

derivatives are predominant in the oil (copaiba oil) and, (3) oils rich in phenylpropanoids and phenolic compounds and other non-terpene oxygenated compounds (anise oil). Figure 3.1 shows typical chromatographic profiles obtained and major components of the EOs isolated by hydrodistillation from three aromatic plants of genus *Lippia*

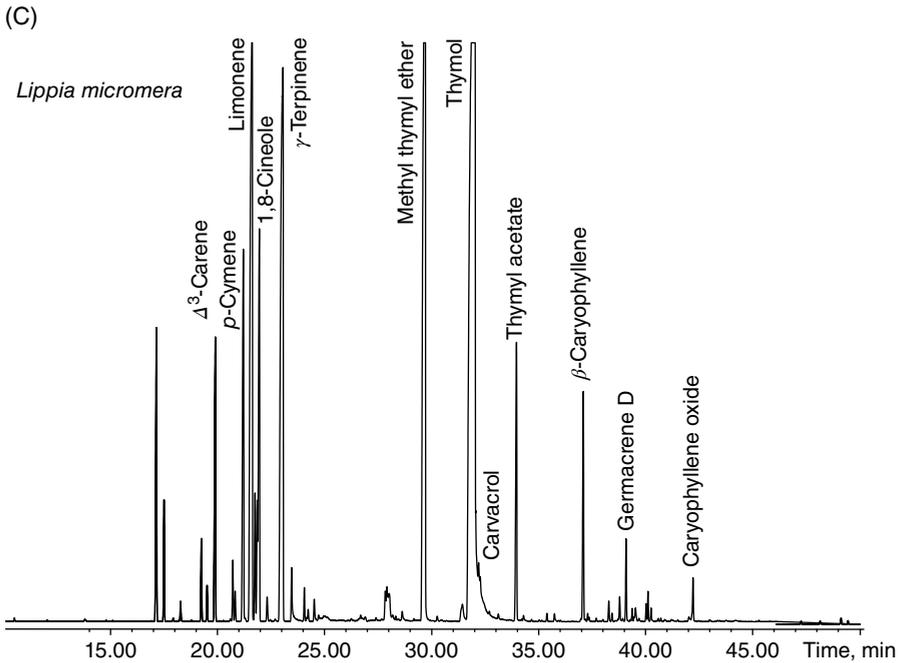


Figure 3.1 (Continued)

(Verbenaceae family), that is, *L. alba* (Carvone chemotype), *L. dulcis* and *L. micromera*. These oils belong respectively to the three different types, namely: (1) Monoterpenoid; (2) Sesquiterpenoid, and (3) Phenolic, according to their predominant components.

3.2.2 Compositional Variation and Dependence on Internal and External Factors

Many factors affect the composition of plant secondary metabolites. The main ones include the geo-climatic location, soil type, stage of plant development (*e.g.*, before, during or after flowering), and even the time of day when the plants are harvested. The geo-climatic factors and soil type can give rise to different plant chemotypes, which are morphologically identical plants with EOs of different chemical composition, sensory properties and biological activity (Figueroa *et al.*, 2008). For example, at least four chemotypes are distinguished in thyme, *Thymus vulgaris* (Labiatae family), according to their main EO compounds: (I) thymol and *p*-cymene; (II) carvacrol, thymol and borneol; (III) linalool, terpinen-4-ol and linalyl acetate, and (IV) geraniol and geranyl acetate. These EOs have different biological properties and smells. While thyme chemotypes I and II possess strong antibacterial activity and are irritant, chemotypes III and IV are not and have moderate antibacterial activity. The EO of thyme chemotype III has a sedative effect due to the presence of linalool, a monoterpene alcohol and its acetate (Thompson *et al.*, 2003). The EOs of geranium plants (*Pelargonium graveolens*) grown in Reunion Island (Indian Ocean, north of Madagascar) or China, constitute another example. In the international market the first EO is known under the name of 'Bourbon' and the second one is called 'Chinese geranium oil'; their chemical compositions are markedly different (Rao, 2009).

Another important parameter is the time of plant harvest. The latter affects both the biological properties and the composition of the extracted oil. For example, sage (*Salvia officinalis*, Labiatae family) EO contains a neurotoxic monoterpene ketone, α -thujone (Cuvelier *et al.*, 1994), in different amounts, depending on the time when the plant is harvested. The content of the ketone is high, when the plant is harvested after flowering and low before flowering. This is precisely the recommended time for the collection of *S. officinalis*. Jasmine flowers, collected in the morning, yield an oil with the preferred combination of linalool, benzyl alcohol, *cis*-jasmone and indole, but when the flowers are collected in the afternoon, their EOs have high levels of benzylbenzoate, methyl salicylate and eugenol (Ahmad *et al.*, 1998); the last two introduce some unpleasant and undesirable odouriferous notes, which can lead to rejection in the perfume industry.

The state of ylang-ylang (*Cananga odorata*, Annonaceae family) flower development, that is, fresh Vs withered, or ripe, yellow Vs green or undeveloped, notably affects oil composition: The extra quality oil is reached amongst other factors, when distilling exclusively fully grown yellow flowers, freshly picked during the early morning hours (Figure 3.2) (Stashenko *et al.*, 1995).

During the circadian cycle, the composition and amount of emitted volatile secondary metabolites of most of the flowers change. For certain plants, for a few hours, the emission of volatiles can be 'suspended' almost completely. Such is the case of the angel trumpet flower ('Datura'), identified botanically as *Brugmansia suaveolens* (Solanaceae family). *Brugmansia* was named in honor of natural history professor Sebald Justin Brugmans (1761–1819); *suaveolens* comes from the Latin, meaning sweet or fragrant smell, the fragrance of its flowers. In our experiments, the volatiles were collected *in vivo* using the technique of solid-phase microextraction (SPME) by exposing the SPME fibre directly to the vapor phase (headspace, fragrance) of the flower (Stashenko & Martínez, 2007) (Figure 3.3). It was established that volatiles emission virtually ceased in the morning, but it resumed during the afternoon, peaked in the early evening and showed a very distinctive circadian cycle.

3.2.3 Essential Oil Isolation and Preparation for Chromatographic Analysis

The chromatographic analysis success strongly depends on careful sample preparation. The analyst must consider the chemical nature of the analytes of interest and the matrix that contains them, to select the appropriate sampling technique. Great advances have been made in miniaturisation, integration, selectivity, efficiency, reproducibility, cost reduction, and robustness of sampling, extraction and concentration techniques. However, certain practises of quality assurance of the analytical chain remain indispensable, such as running blanks, using clean-up steps and doing replicates (Koning *et al.*, 2009). The extracts isolated by SPME or thermal desorption, or the volatile fractions resulting from steam distillation, hydrodistillation or simultaneous distillation-solvent extraction, represent a perfect mixture for analysis by GC-MS. They may only require drying, for example, with anhydrous sodium sulfate, prior to dilution (typically, 1:20–1:30 in dichloromethane) and injection of the sample into the chromatograph. In the situations in which an electron-capture detector is employed, a non-halogenated solvent, such as acetonitrile, should be used. A very useful sample preparation guide for GC analysis is that of Koning *et al.* (2009).

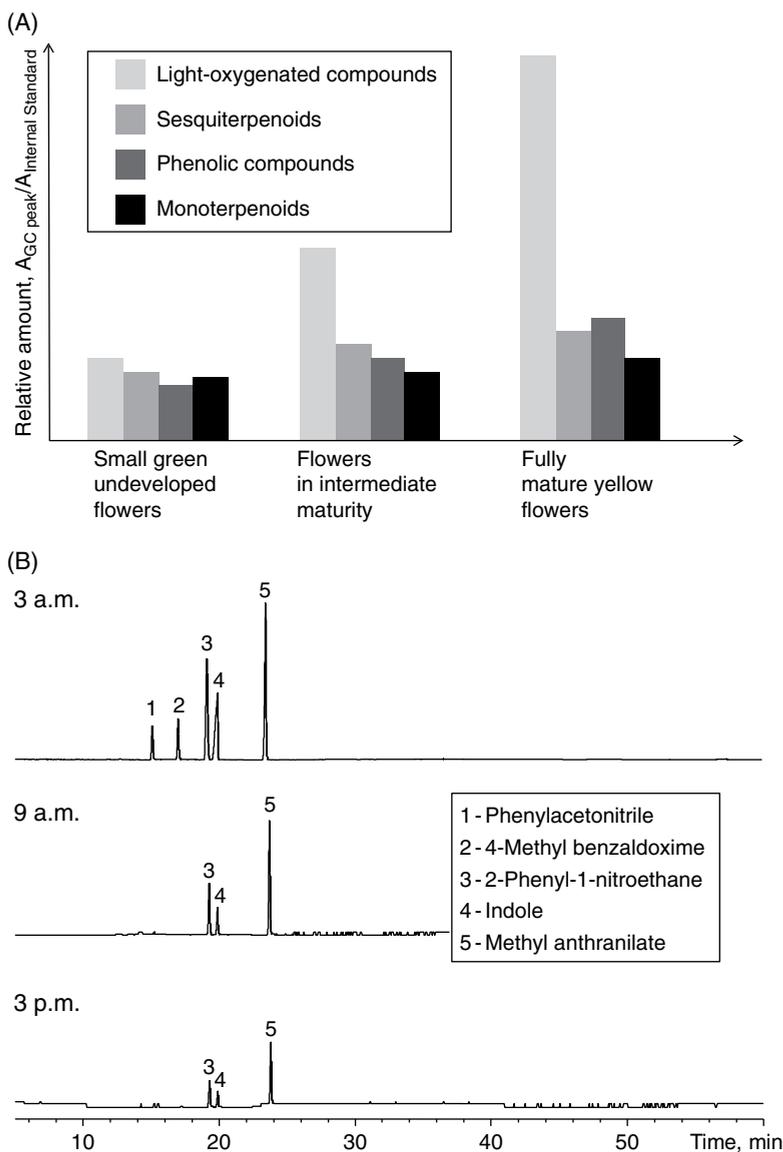


Figure 3.2 A. Compositional variation of ylang-ylang (*Cananga odorata*, Annonaceae family) essential oils obtained by hydrodistillation from flowers at different stages of their development. High-quality essential oil could be obtained from yellow fully mature flowers picked during the early morning. **B.** Variation of nitrogen-containing compounds (GC-NPD) in ylang-ylang essential oils obtained by hydrodistillation from mature flowers gathered at different collection times.

Microwave radiation has been used in the past 30 years for heating water (microwave-assisted hydrodistillation, MWHd), to increase the efficiency of the extraction as well as to shorten drastically the distillation time. It has also been used to heat an organic solvent or the sample, if the dielectric constant, ϵ , of one or the other is sufficiently high (Rice & Mitra, 2007). Microwave-assisted extraction (MAE) methods have found many

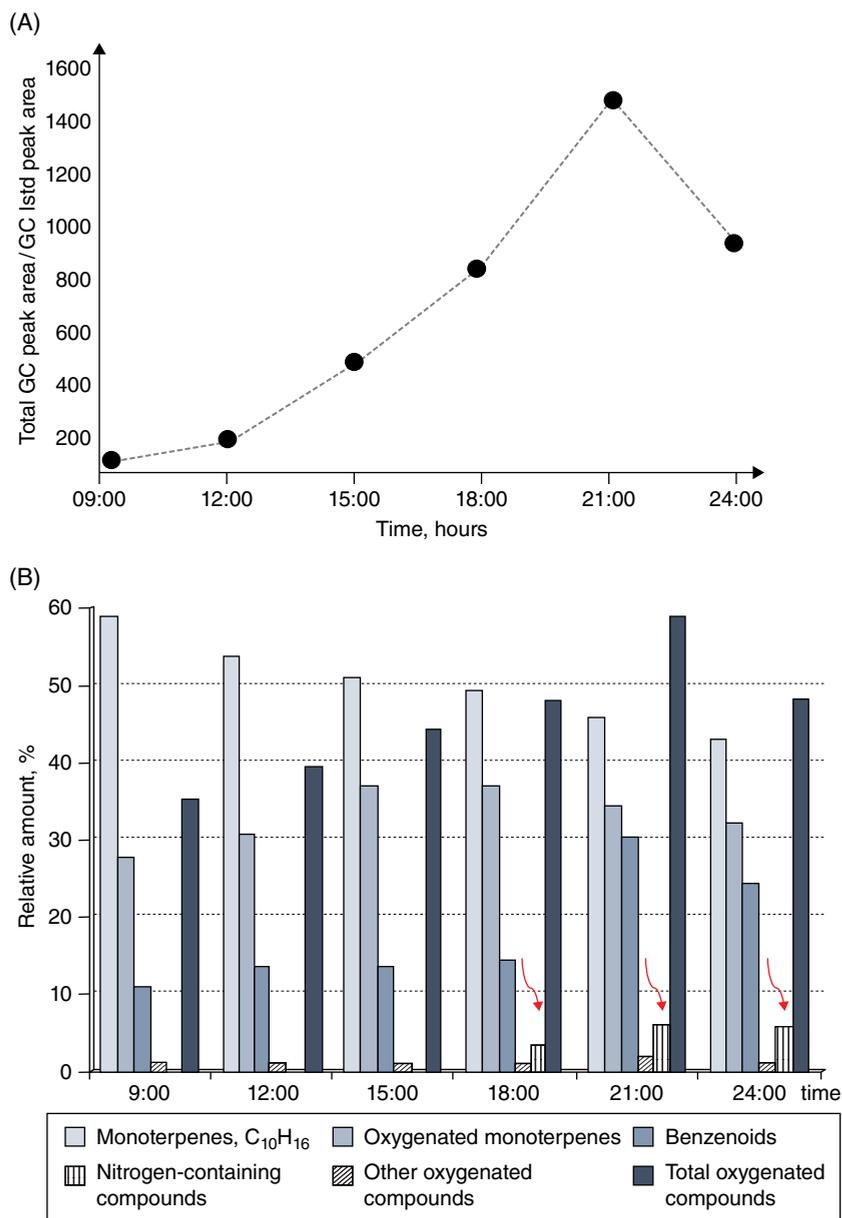


Figure 3.3 *In vivo* HS-SPME-GC/FID/MS analysis of the volatile fractions from Angel trumpet flowers (*Brugmansia suaveolens*, Solanaceae family), monitored at different times. **A.** Volatile emission during the day measured as a total GC peak area with respect to that of the internal standard (lstd, *n*-tetradecane); **B.** Volatiles produced by flowers at different times, classified per compound families. The flowers emit nitrogen-containing compounds mostly during the night time.

applications in the extraction of solids (vegetal material, soil, tissue, *etc.*) with either water or an organic solvent (Kingston & Haswell, 1997).

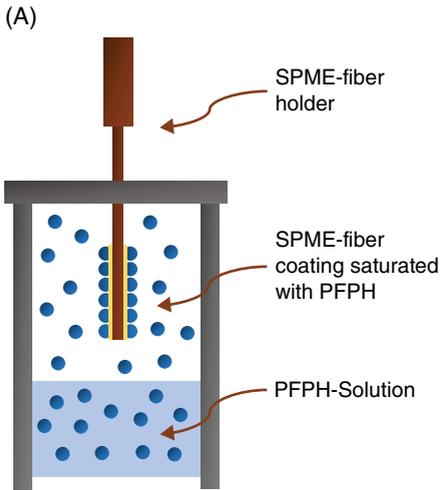
Solid-phase microextraction is already in its third decade of development and wide application, particularly, in volatile substances sampling. It combines analytes'

extraction and concentration and can be used in three modes: (1) in headspace, (2) by direct contact (immersion) of the fibre into the fluid sample and (3) by using a membrane between the sample and the fibre (Pawliszyn, 2012). It is very well-suited to the analysis of compounds by GC-MS and the sequences of steps (SPME fibre conditioning, exposure and desorption) is now performed automatically by several commercial devices. The SPME method selectivity may be modified by (1) varying operating conditions (temperature, salting-out, fibre exposure mode, agitation and fibre coating type, amongst others), (2) using selective chromatographic detection systems (electron-capture detector, ECD; flame-photometric detector, FPD; nitrogen-phosphorus detector, NPD; mass spectrometer in selected ion monitoring mode, MS-SIM, *etc.*), and (3) modifying the target analytes in the matrix or by derivatisation with a reagent previously loaded on the fibre (Risticvic *et al.*, 2010). A very interesting SPME application which affords selectivity during sampling is the extraction with analyte derivatisation (1) in the matrix, (2) in the fibre, saturated with a derivatising agent (Figure 3.4) or (3) directly into the GC injection port (Stashenko & Martínez, 2004).

3.3 Essential Oil Component Identification

EO chemical characterisation is based on its gas chromatographic profile, obtained under various chromatographic conditions (oven temperature programme, gas flows), injection modes (split, direct on-column, pulsed) and with different detection systems. Mass spectrometry is the preferred detection system for the identification of EO components. In very specific cases, such as with new (unknown) compounds, or when geometric isomers should be distinguished, it may be necessary to completely isolate one of its components to obtain additional information from spectroscopic techniques such as nuclear magnetic resonance (Sparkman *et al.*, 2011).

Infrared (IR) and Raman spectroscopy are complementary vibrational spectroscopy techniques that can be employed to distinguish individual EO components or to compare different EOs. Siatis *et al.* (2005) used multivariate statistical techniques in the quantitative analysis of thymol, carvacrol, *p*-cymene and γ -terpinene in oregano and thyme EOs. The procedure was possible because the Raman spectra of these substances contain individual, distinctive signals for each one of them, which can be detected or resolved in the EO Raman spectrum. This principle was used by Daferera *et al.* (2002) to recognise the main components of several EO from the Lamiaceae family. A partial least squares regression model based on Raman spectroscopy data permitted the quantitative analysis of linalyl acetate, linalool and eucalyptol in lavandin or lavender EOs (Lafhal *et al.*, 2015). The quantitation procedure was calibrated with GC data. Schulz and Baranska (2007) reviewed the subject of valuable plant substances identification using vibrational spectroscopy. They provided examples of cases in which characteristic Raman and IR bands of individual substances permitted their quantification in the EO. It is possible to use mass spectrometry in an analogous manner. The mass spectrum of the whole, unfractionated EO, can be used to identify and quantify certain components, if specific signals associated with them are discernible or can be resolved using chemometric methods. Although the sensitivity of NMR is much lower, similar applications have been developed with this spectroscopy for the detection and quantification of EO components (Tomi & Casanova, 2006). However, the characterisation of all the EO components does require a chromatographic step, their separation, before obtaining the spectral/structural data.



(B)

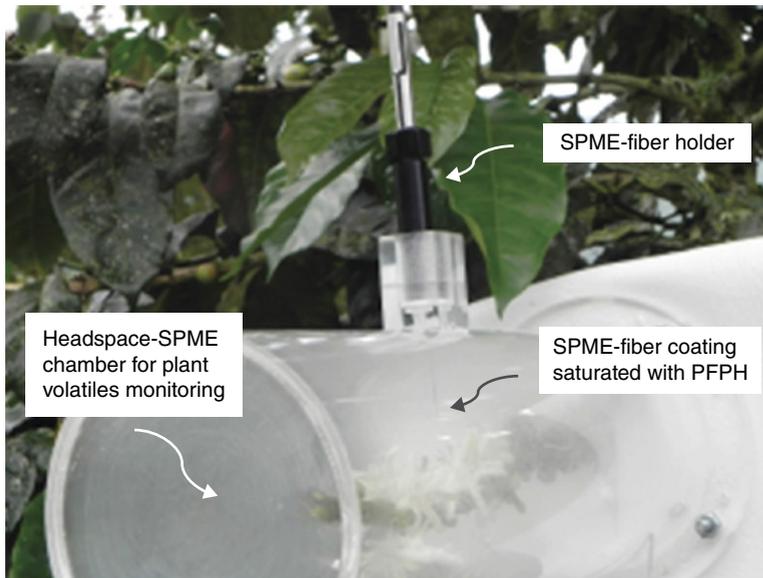


Figure 3.4 **A.** SPME-fibre saturation with the derivatising agent (PFPH, pentafluorophenyl hydrazine); **B.** Set-up for *in vivo* headspace-SPME monitoring of plant volatiles. **C.** Chromatogram obtained by GC- μ -ECD (micro-electron capture detection) of carbonyl compounds (detected as their hydrazones), isolated from *Swinglea glutinosa* fruits (Rutaceae family) by *in vivo* HS-SPME, using the fibre saturated with PFPH-derivatising agent.

An initial screening analysis employs absolute (t_R) or relative (t_{RR}) chromatographic retention times and certified standards for presumptive compound identification by GC with non-spectrometric detectors (flame ionisation detector, FID). Confirmatory identification of a compound in a complex mixture, analysed by GC, necessarily requires obtaining its unique spectral data. In addition to the retention times or retention

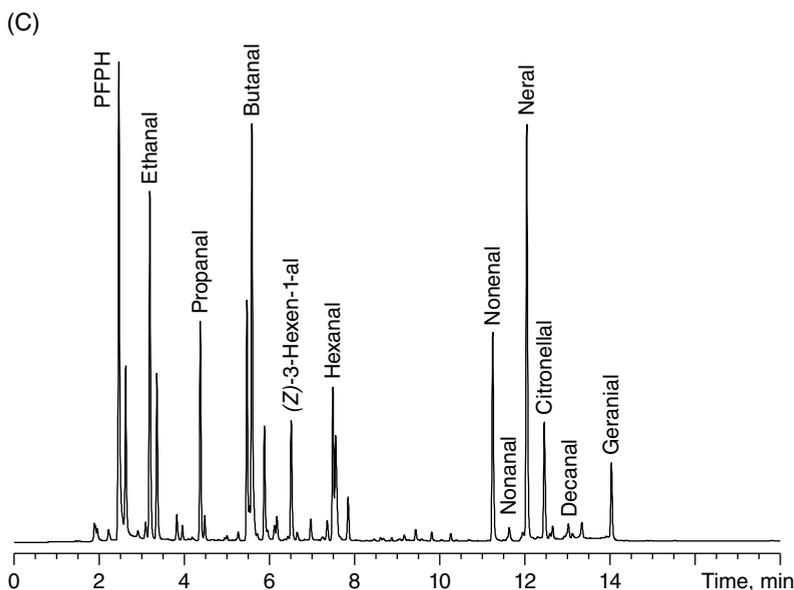


Figure 3.4 (Continued)

indices obtained by GC, the mass spectrum discloses the relative amounts of a unique group of charged fragments (ions) generated during dissociation or fragmentation of the molecule after its ionisation. In the case of high-resolution mass spectrometry, the data permit the exact calculation of the isotopic composition of each molecular fragment (Kandiah & Urban, 2013).

The GC-MS identification of an EO component requires several steps. Chromatographic profiles on both polar and non-polar stationary phases should be obtained, the retention indices (Section 3.3.2) should be calculated, and the mass spectra (electron ionisation, EI, 70 eV) should be acquired. These data are then compared with those of databases, abstracts, books or articles (Adams, 2007; Babushok & Zenkevich, 2009; Davies, 1990; Jennings & Shibamoto, 1980; Joulain & König, 1998) or of standard compounds analysed under the same operation conditions. The coincidence of the experimental parameters with those of the references leads to structural recognition of the substance, but if this agreement includes the linear retention indices in both types of stationary phases (non-polar and polar) and the mass spectrum of the certified substance standard, a positive identification, and a structural confirmation, is obtained (Molyneux & Schieberle, 2007). Section 3.4.3 describes the application of multidimensional and comprehensive chromatographic techniques to EO characterisation. Basically, the same requirements should be satisfied for structural confirmation, but comprehensive chromatography performs the EO separation on polar and non-polar stationary phases in a single experiment.

3.3.1 Gas Chromatography

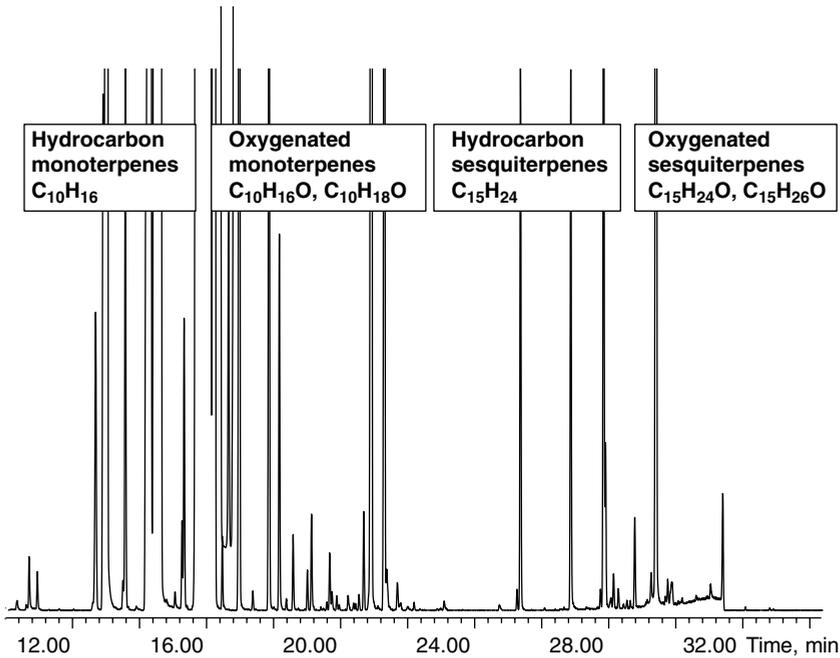
Gas chromatography is a technique widely applied in many branches of science and technology. The ability to establish the nature and chemical structure of the separated and quantified compounds depends strongly on the detection system employed. The

mass spectrometric detector is the most used system. Besides the structural information that it provides, its high sensitivity permits the detection of substances at trace amounts. High-resolution mass spectrometry (HRMS) increases the accuracy of the molecular weight and elemental composition determination. When chiral columns and standard compounds are used, the optical isomers can be determined (Bicchi *et al.*, 1999). This is very important for establishing EO authenticity.

In GC, the separation of the EO components is based on their partition between the inner coating of the chromatographic column and the gas phase. The ineludible step to obtain this situation is the volatilisation of the sample. This takes place at the chromatograph's injection port, which is maintained at or above 200 °C. Upon exposure to the injection port temperature, the EO liquid solution or the substances on the SPME fibre coating, pass to the gas phase. The carrier gas (N₂, He or H₂) transports these volatilised substances to the chromatographic column. These substances interact with the thin polymer film (of polar, non-polar or intermediate nature) that coats the inner walls of the fused-silica capillary column (~0.2 mm inner diameter). The strength of this interaction determines how much a particular analyte molecule is delayed during its travel through the chromatographic column. The carrier gas stream (mobile phase) and the column's inner polymeric coating (stationary phase) are the two phases between which the analyte molecules are partitioned. This is a temperature-dependent process. The temperature of the chromatographic column is changed during GC operation to improve the separation. An electric signal which is proportional to the amount of substance or its concentration is obtained at the end of the chromatographic column by means of a detector. Physical properties such as thermal conductivity, or physicochemical processes such as electron capture or the ionisation in a flame, are phenomena on which detection systems are based. A data system is used to collect, store and process the detector signal. The chromatographic profile, or chromatogram, is a graphical representation of the change of the detector signal with time (Figure 3.5).

The chromatograph has four principal sections: introduction, separation, detection and data handling. The quality of the chromatographic results depends on the appropriate operation of each one of these sections (Grob & Barry, 2004). The sample should be transferred quantitatively to the column by the injection system, without discrimination due to molecular weights or vapor pressures, and without chemical modification (decomposition or isomerisation). This step is critical, particularly for quantitative analysis. A clean, inert and leak-free conduit from the point of sample admission to the chromatographic column is crucial for correct GC operation. A GC-suitable analyte should be sufficiently volatile as well as thermostable so that its amount in the mobile phase is detectable. If the analyte's volatility is very low it will not enter the chromatographic column. It will not be transferred to the carrier stream, will accumulate at the injection port and may eventually clog it. Therefore, analytes not amenable to GC analysis include ionic or very polar substances, thermolabile or high molecular-weight compounds. A chemical derivative with higher volatility, which is thus more suitable to GC analysis, may be obtained in some cases. The books by Blau and Helket (1993) and by Toyōoka (1999) are important guides to the selection of derivatisation reagents and reaction conditions. However, the techniques employed for their production (steam distillation, hydrodistillation) ensure that EO components are sufficiently volatile for GC analysis.

(A)



(B)

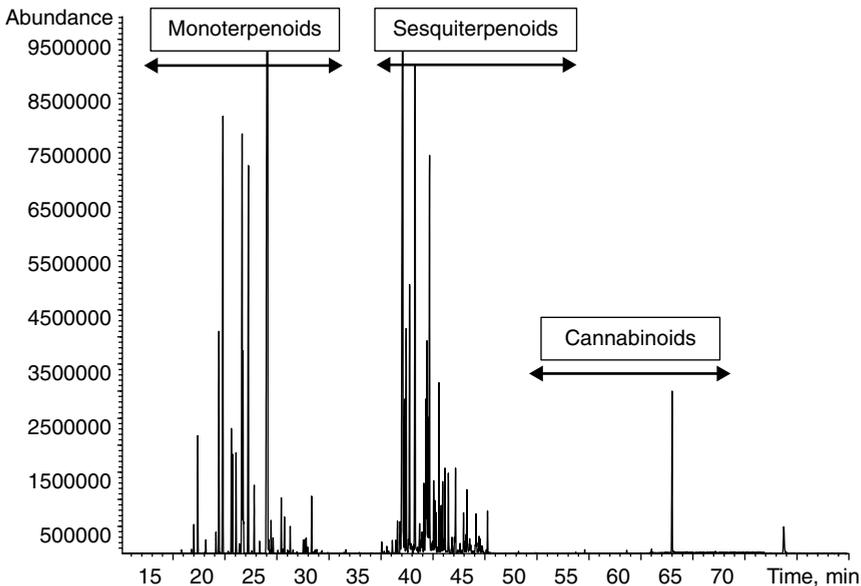


Figure 3.5 A. Typical chromatogram obtained by GC-FID of the essential oil hydrodistilled from *Swinglea glutinosa* (Rutaceae family) fruit peels. Non-polar (PDMS) 30-m-capillary column. Characteristic elution order according to the component retention time increase, as follows: hydrocarbon monoterpenes → oxygenated monoterpenes → hydrocarbon sesquiterpenes → oxygenated sesquiterpenes. B. Typical chromatogram (TIC) obtained by GC-MS (electron ionisation, 70 eV) of the essential oil hydrodistilled from *Cannabis sativa* inflorescences. Three compound families, that is, monoterpenoids, C_{10} ; sesquiterpenoids, C_{15} , and cannabinoids, C_{17-21} are clearly distinguishable.

The distribution constant of an analyte between the mobile and stationary phases is K_D . The resolution of a chromatographic column is its capacity to separate substances with very close K_D . The type of stationary phase, the mobile phase, temperature, the column dimensions [length (L), inner diameter (ID) and stationary phase thickness (d_f)], are those operational parameters which can modify the resolution obtained in a chromatographic run. As the sample complexity increases (number of components and structural similarity) complete compound separation requires the use of longer columns or columns with smaller diameter. Using smaller internal diameter columns leads to lower sample capacity (detectability drops). Obviously, column length elongation is accompanied by longer analysis times. The analysis of polyaromatic hydrocarbons (PAHs), steroids, pesticides or controlled drugs is accomplished satisfactorily using 30 m-long column, but the separation of hydrocarbons in gasoline requires longer, 100 m-column. Most EOs can be analysed on 50 or 60 m-column.

3.3.1.1 Columns

Since EOs may be formed by many components (tens to hundreds) and, in addition, many of them may be isomers (position, geometric, stereoisomers), long chromatographic columns (50 to 60 m) are usually employed for these analyses. The column inner diameter is chosen per the amount of sample to be injected, but typically, columns of 0.25 or 0.32 mm ID are used, although columns of smaller diameter (0.18, 0.20 or 0.22 mm) allow achieving a much higher separation efficiency, although compromising, in some cases, the sensitivity (column capacity). An important parameter is the thickness of the stationary phase (d_f), which, for chromatographic analysis of volatile compounds must be greater (typically, columns of 0.25 or 0.33 μm , d_f are used) and, for very volatile analytes, can reach up to 5 μm . Since analyte polarity determines the choice of stationary-phase polarity and EOs contain both polar and nonpolar analytes, it is necessary (required) to use both, that is, polar (polyethylene glycol) and nonpolar [100%-poly(dimethylsiloxane) or 5–95%-phenyl-poly(methyl siloxane)] columns.

For volatile compound analysis, an initial column temperature of 35–50 °C could be recommended; the nature of the sample (EO) does not require a high final column temperature. Thus, 200–250 °C is sufficient to elute the most retained EO components. The heating rate of the column is a function of its length: the longer column should be heated more slowly, 3–4 °C/min, but if shorter columns are used, for example, 30 m, the column temperature could increase quicker, at a rate of 5–7 °C/min or faster. Of course, the temperature-programming process is optimisable, depends on the complexity of the mixture of substances to separate (number of components, their structural similarity, isomerism), their nature (polarity, molecular weight), the column dimensions (L , ID), the thickness (d_f) and the stationary phase type (polarity).

The separation in the chromatographic column is based on achieving different distribution constants, K_D , of the components between the stationary and mobile phases. The tools available to achieve separation of mixture components include temperature modification, column head pressure, mobile phase speed, stationary phase polarity, correctly choosing the chromatographic column dimensions (L , ID), stationary phase type, its chemical composition, thickness, amongst other factors. Increasing two-fold the column length doubles the resolution, while reducing the column internal diameter by two times increases the resolution four times. The reduction in column diameter to 0.1 or 0.05 mm (micro-bore columns), led to the so-called fast chromatography, which

can separate complex mixtures in seconds. However, it also resulted in a dramatic reduction in sample capacity; that is, trace level compounds cannot be determined with so narrow-ID columns. Selecting the length and internal diameter of the column is a very important compromise between analysis time, the required resolution, and sensitivity (Hinshaw & Ettre, 1994). Sample volatility is the main parameter to choose the stationary phase thickness. Columns with greater thickness, up to 5 μm , are required for mixtures of volatile compounds. High-boiling point compounds, for example, polyaromatic hydrocarbons, steroids or diterpenes, are separated in columns with smaller d_f , for example, 0.25 μm or less. The increase of the stationary phase thickness leads to greater compound retention, which demands higher column temperature to accelerate components elution, but it also leads to greater bleeding of the stationary phase, that becomes more pronounced at higher temperatures in most columns. In other words, volatility (vapor pressure) of substances is the best guide for the correct selection of the stationary phase thickness: for higher vapor pressure (lower boiling point) components, larger d_f values are required.

What stationary phase polarity to employ depends on the sample's chemical nature, the polarity of the mixture components and their relative amounts. In non-polar columns, for example, poly(dimethylsiloxanes), generally, the compounds elute according to their boiling points; in polar columns, for example, poly(ethylene glycol), the intermolecular forces (between polymer and analyte) and the substance dipole moment govern retention and elution order from the column. The analysis of complex mixtures, containing both polar and non-polar components, is performed in two columns of different polarity (Scott, 1998). Figure 3.6 shows two typical chromatograms of EO isolated from *Pogostemon cablin* (Labiatae family) examined in two columns of different stationary phase, polar (polyethylene glycol) and nonpolar (polydimethylsiloxane). Retention times (t_R) of the same components change with the change of stationary phase polarity.

3.3.1.2 Injection Systems

Virtually, all kinds of injection ports can be used for chromatographic analysis of a volatile substances mixture. The classic split/splitless injection system is commonly used in EO analysis. The injection port temperature must be sufficiently high to vaporise all mixture components, but should not exceed their decomposition temperatures. For volatile compound analysis, the inlet temperature maybe in the range of 200–250 °C. Preliminary tests may permit to find the temperature that provides greater sensitivity (total chromatographic area) (Figure 3.7A). When the injection port is used mainly in the split mode, the analyte amount that enters the GC column should be sufficient and higher than the minimum detection levels. Splitless mode pulsed injection is a good option for compounds present at trace levels, but at the same time, for the thermally unstable ones (Grob, 1993). Programmed-temperature injectors (PTV), amongst other benefits (*e.g.*, large volume injection), allow to concentrate analytes *in situ* in the extract, thanks to a programmed solvent evaporation. The on-column injectors are suitable for both highly volatile substances (with a cryo-focusing device) and for marginally volatile or thermally unstable substances (Grob, 1987).

When SPME is used, it is important to replace the liner for one with smaller dimensions, designed to reduce peak broadening. For many applications that involve retention time and chromatographic area measurements, the steady and reproducible operation of the injection system should be assured (Figure 3.7B). To accomplish this,

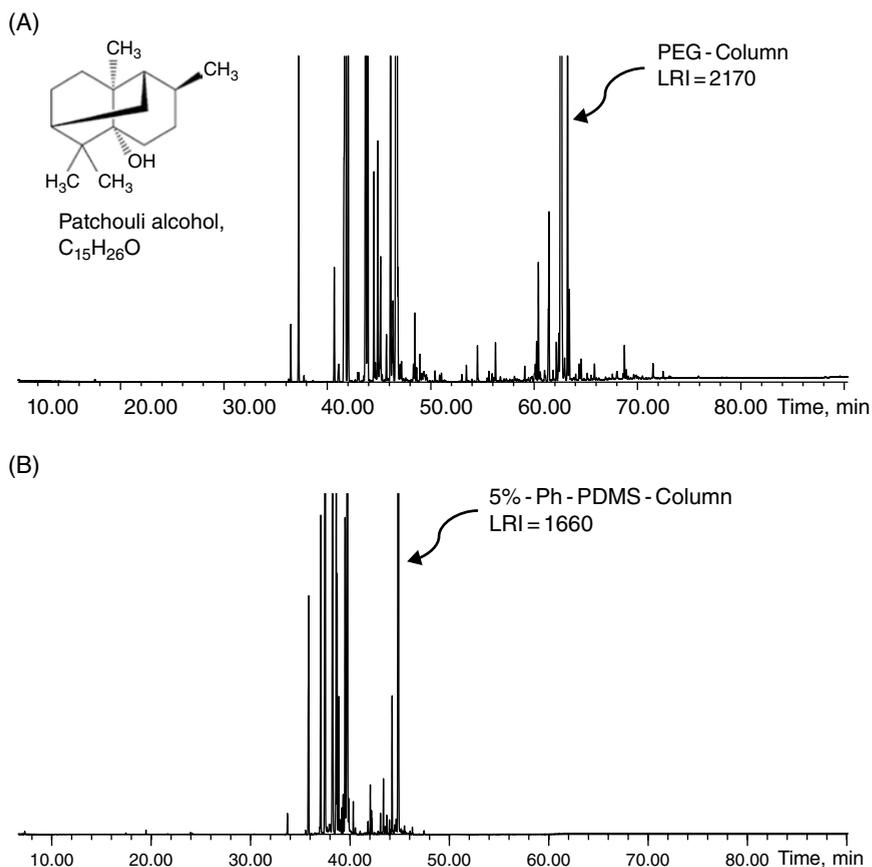


Figure 3.6 Typical chromatograms of steam-distilled *Pogostemon cablin* (patchouli, Labiatae family) essential oils, obtained with GC-FID on: **A.** Polar column (60 m x 0.25 mm), coated with poly (ethyleneglycol), PEG; and **B.** Non-polar column (60 m x 0.25 mm), coated with 5%-phenyl-poly(dimethylsiloxane). For the same compound, for example, patchouli alcohol, linear retention indices (LRI) change notoriously with the stationary phase polarity.

the same sample is injected into the column several times (the same day and on different days or weeks) and standard deviation or coefficient of variation (CV%) of these measurements is determined. The retention time can also depend on the amount of substance injected (Figure 3.7C).

3.3.1.3 Detection Systems

The detection system generates a signal upon the presence of the analyte molecules but no signal from the carrier gas molecules. Refractive index, fluorescence, ionic current, thermal conductivity and photon emission are examples of physical properties that can be used to generate a detector response. Analyte quantitation requires a direct proportionality between detector signal strength and the number of analyte molecules emerging from the chromatographic column. The mass selective detector operated in the full scan mode, the thermal conductivity detector (TCD), or the infrared detector (IR) are classified as universal detectors. This is because they generate a signal for almost any

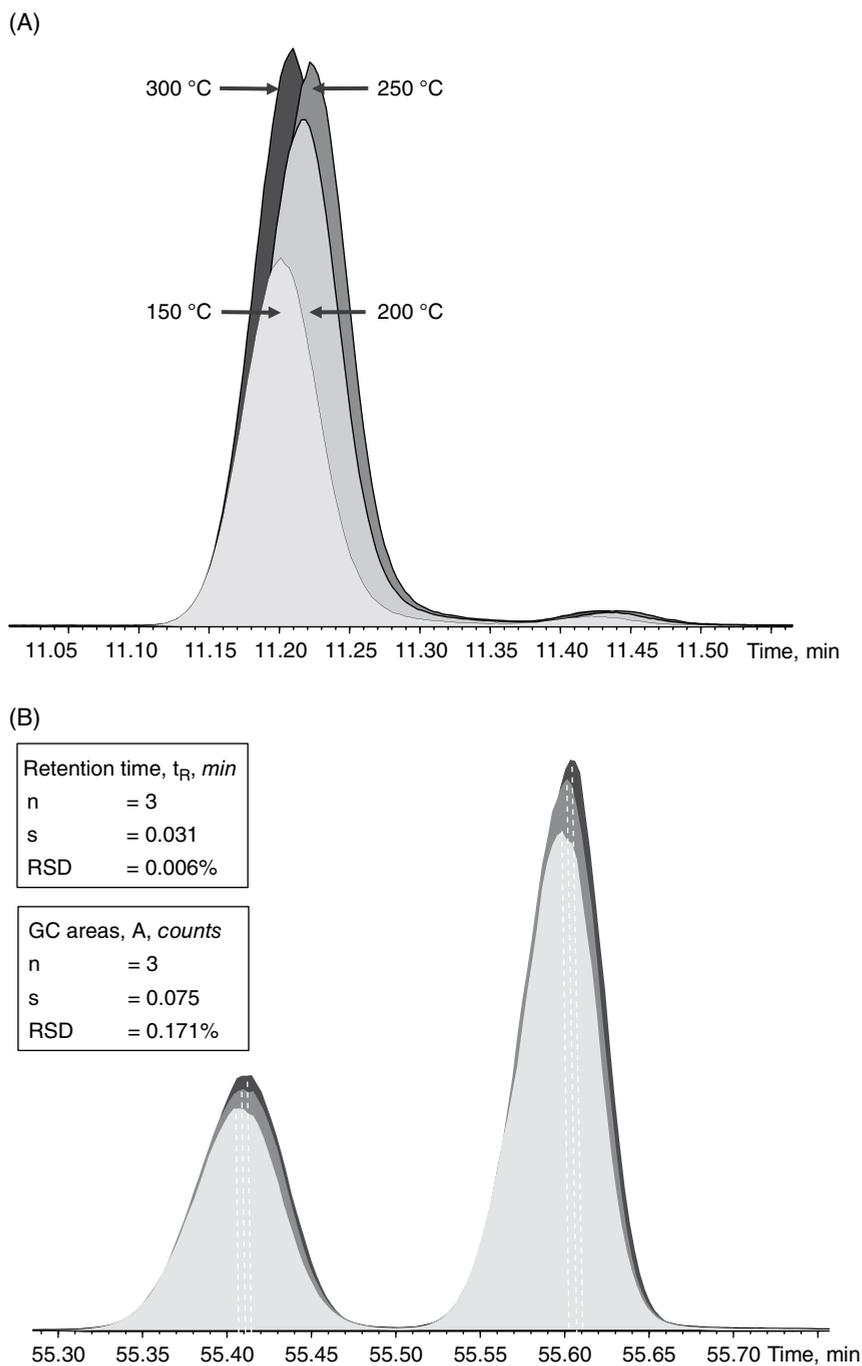


Figure 3.7 A. Dependence of the GC peak area of a compound on the injection port temperature. B. Repeatability of GC retention times and GC peak areas when injecting the same compound several times ($n=3$, s – standard deviation and RSD – relative standard deviation). C. Retention time dependence on the amount of compound injected.

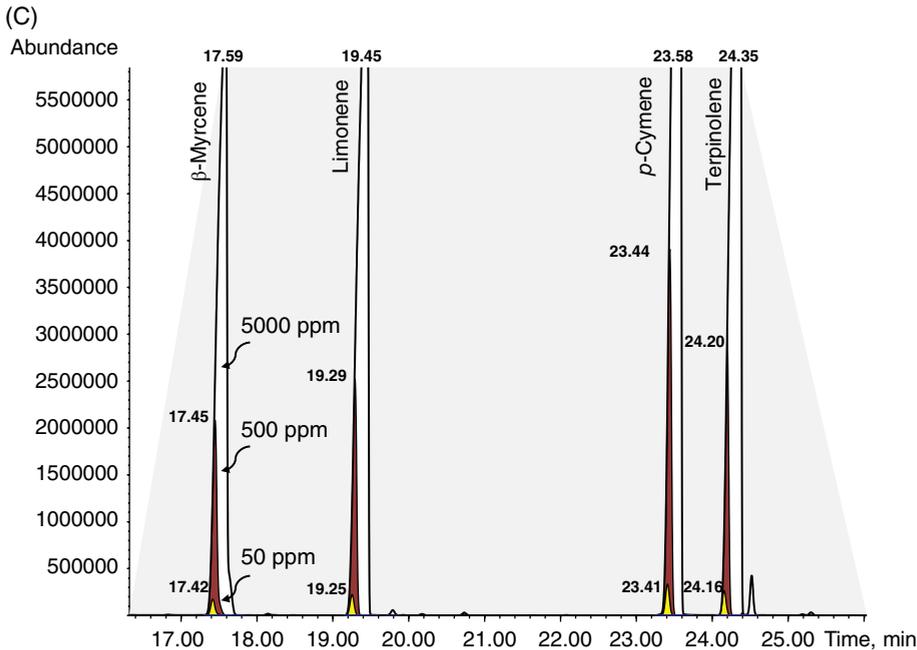


Figure 3.7 (Continued)

substance. There are detectors whose signal depends on the existence of features in the analyte's structure: the nitrogen-phosphorus detector (NPD), the electron capture detector (ECD) and the flame-photometric detector (FPD) are examples of these selective detectors. Specific detectors are the third class of gas chromatographic detectors. When MS operates in selected ion monitoring (SIM) mode (Figure 3.8, *Moringa oleifera* EO), or in the case of the triple quadrupole detector in single reaction monitoring mode, the signals originate only upon the presence of a set of ions, which can result only from specific molecules. The thermal energy analyser (TEA), and the atomic emission detector (AED) are additional examples of specific detectors (Dressler, 1986).

The FID could be treated as 'near-universal', because it is unresponsive only to water and permanent gases. The border between selective and specific detectors is well-defined. Specific detectors are highly selective detection systems that provide a signal to a compound, present in a complex mixture (Figure 3.9).

Examples of the specific features on which the signal of a selective detector is generated are the presence of an electronegative atom, such as nitrogen, phosphorus (NPD) or sulfur (FPD), or the existence of an electron-attracting functional group (ECD) or multiple bonds or aromatic rings (PID, photoionisation detector) or a structural fragment common to groups of substances, such as a phenyl, benzyl or an acyl radical, and so on (MS operated in SIM mode; Figure 3.10).

Linearity, limits of detection and quantification, sensitivity and susceptibility to changes in gas flow, temperature or pressure are parameters that permit to differentiate chromatographic detectors. GC detector choice and operation should consider their noise level, volume, strength and sophistication, simplicity and operation cost, amongst other typical characteristics. The response of most GC detectors depends on analyte

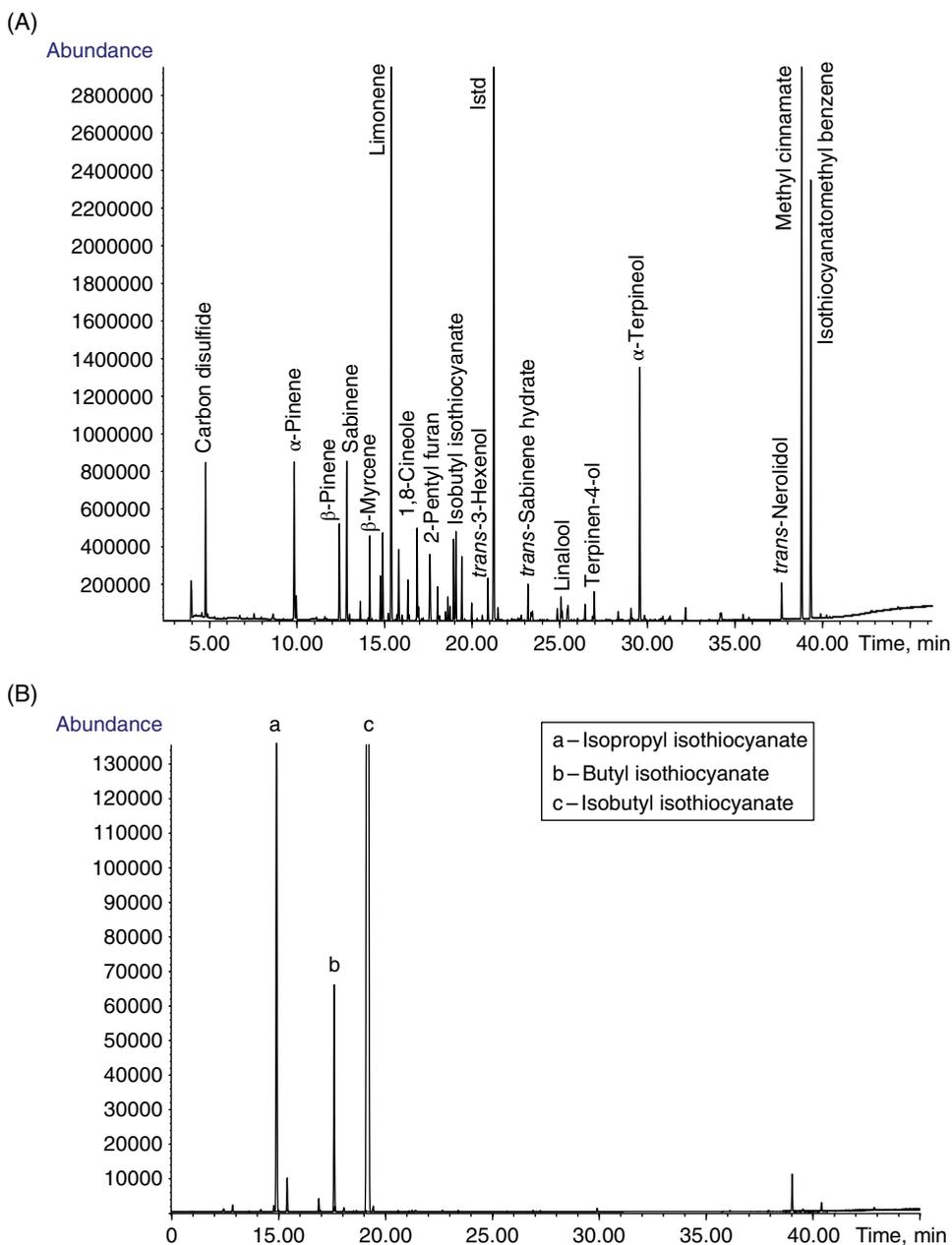


Figure 3.8 **A.** Total ion current (TIC) obtained by GC-MS (electron energy, 70 eV) of the volatile fraction isolated by *in vivo* HS-SPME (PDMS/DVB-fibre) from *Moringa oleifera* flowers (Moringaceae family). **B.** Extracted ion chromatogram (EIC) based on the 'diagnostic' for isothiocyanates fragment-ion (m/z 101 and m/z 115) currents.

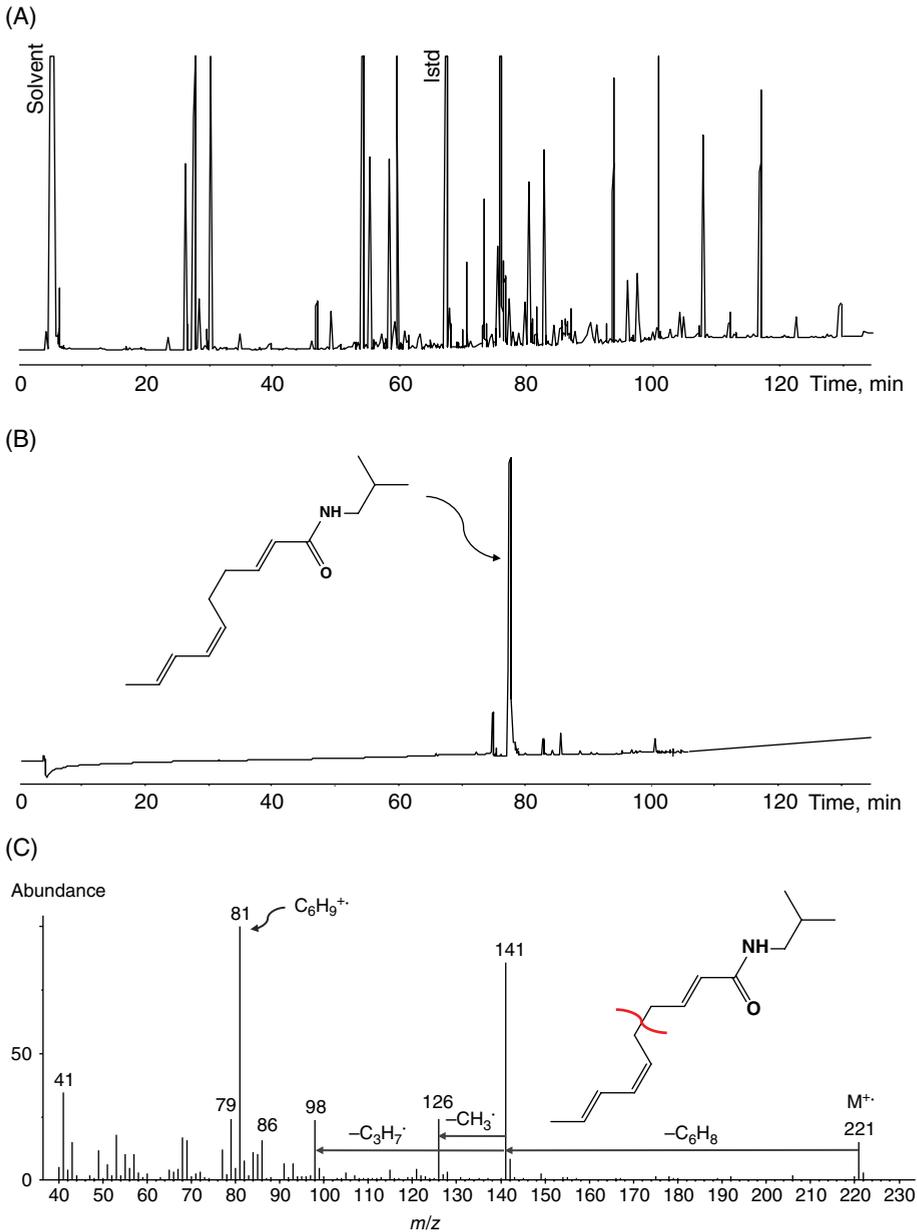


Figure 3.9 **A.** Typical chromatogram obtained by GC-MS (total ion current, TIC, EI, 70 eV, full scan) of *Spilanthes americana* (Asteraceae family) hydrodistilled essential oil. Internal standard, Istd, *n*-tetradecane; **B.** Chromatogram of the *S. americana* essential oil, obtained with the nitrogen-phosphorous detection system (NPD), showing spilanthol [(*2E*, *6Z*, *8E*)-*N*-isobutyl-2,6,8-decatrienamamide, $C_{14}H_{23}NO$] as the main nitrogen-containing compound; **C.** Mass spectrum and principal fragment-ions of spilanthol, registered at 70 eV-electron energy.

mass changes (FID, NPD, FPD, MSD). These GC detectors are destructive. On the other hand, the signal of non-destructive detectors (TCD, IR detector; ECD, PID) is sensitive to the change of analyte concentration. In the operation of TCD or ECD, it is important that the

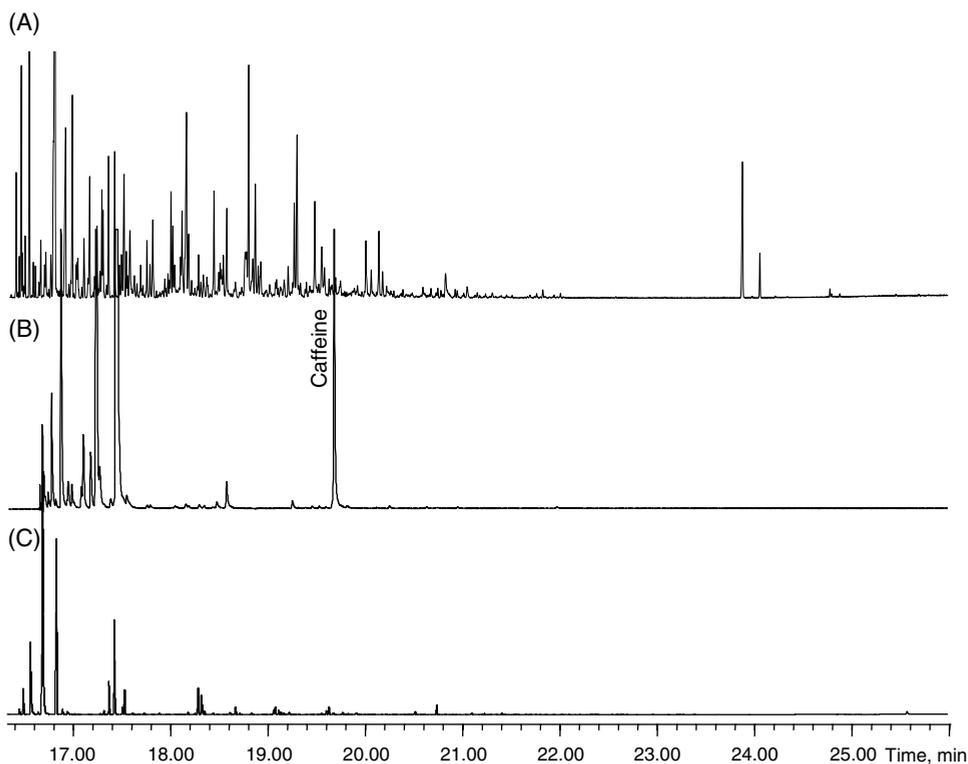


Figure 3.10 Gas chromatographic analysis of the volatile fraction obtained by purge-and-trap (P&T) method from toasted coffee grains, using different detection systems, as follows: **A.** Mass selective detector (MSD) operated in full scan mode; **B.** Nitrogen-phosphorous detection (NPD) and **C.** Flame-photometric detection (FPD). These detection systems register all substances, only nitrogen-containing compounds or sulphur-containing compounds, respectively, in the coffee volatile fraction.

gas flows (carrier gas and make-up or auxiliary gases) are maintained constant. Non-destructive detectors can be arranged in series and can precede the destructive detectors.

The type of GC detector employed determines, firstly, the purpose of the analysis and, above all, the nature of the compounds to determine. Basically, a selective detector is used when the 'analytical lens' is focused on only one or a few analytes particularly present in a complex mixture, to register (*e.g.*, nitrogen or sulphur compounds) and quantify them, 'ignoring' the presence of other mixture constituents. In a complex mixture of volatile substances isolated by dynamic headspace (inert gas purge and trap solvent, CH_2Cl_2) of roasted coffee beans, some nitrogen compounds, for example, pyrazines, pyridines, oxazoles and Maillard reaction products may have an analytical and sensory interest. These substances are selectively detected by the NPD; sulphur-containing compounds can be selectively detected by FPD (Figure 3.10).

3.3.2 Retention Indices

In gas chromatography, the separation of substances is based on differences in their distribution constant, K_D , between the mobile and stationary phases. The chromatographic 'art' (*i.e.*, the combination of a basic knowledge, practical experience, and

intuition) comprises the proper selection of thermodynamic conditions of the separation process, leading to the situation when the K_D for different substances, obtained under unique operational conditions, become different. This, unfortunately, is not achieved in some cases. K_D depends on the chemical nature of the mobile and stationary phases and of the analyte as well as on the column temperature. Analytes retention in a column is a function of their vapor pressures and intermolecular forces that govern their interactions with the stationary phase. K_D affects the rate of analyte elution, or what is more commonly known and used, the so-called retention times, characteristic for a given chromatographic column and for operating conditions. If the average linear speed of the carrier gas in the column is constant, the retention of analytes is basically influenced by the length of their stay in the stationary phase. Higher K_D values imply longer retention times. The latter, however, depend on multiple operational variables, namely: the type and dimensions of the column, the polarity of the stationary phase and its thickness, column temperature and heating rate, the type of carrier gas and its speed, the injection mode and the column head pressure and also hinge on the amount (concentration) of the analyte injected into the column (Figure 3.7C), the degree of use (*i.e.*, wear), pollution, or activation, of the latter (Laskin & Lifshitz, 2006).

Generally, for both FID and MSD systems, t_R reproducibilities are high ($CV < 0.2\%$). However, the retention times are very sensitive to changes in chromatographic conditions. Since its beginnings and throughout its development, GC analysis has always had the need to 'liberate' retention parameters from operational chromatographic conditions, to have a tool or parameter that can be compared, which may serve for prior identification or differentiation of analytes in GC or to compare intra- and inter-laboratory data. The determination of the relative retention times, t_{RR} , based on the use of an internal standard, in part, helps to minimise t_R dependence on some operational parameters, for example, temperature fluctuation or carrier gas, activation sites in the column, and so on, but it does not help to compare the inter-laboratory data or to perform the tentative identification of substances (compared to their certified standards). It is also very difficult for a chromatographic run of a multicomponent mixture to find the unique internal standard that fits all substances in the mixture, and elutes in a wide range of t_R .

To increase the degree of reliability in comparing the chromatographic retention data and allow the tentative identification of the analytes, more than half a century ago, the Hungarian scientist, E. Kovàts introduced a system of retention indices, called 'Kováts indices' (KI), based on measurement of the relative retention times with respect to a homologous series of *n*-paraffins, that is analysed under the same chromatographic conditions as the sample (Kováts, 1965). In the late 1950s of the twentieth century, measurements were carried out at constant temperature (isothermal regime); a few years later, commercial chromatographs were capable of reproducibly changing the temperature of the chromatographic oven.

When the analysis is performed in isothermal regime, the logarithm of the retention time ($\log t_R$) increases with the number of carbon atoms in the *n*-paraffin; by definition, retention rates were assigned by E. Kovàts as follows: for example, 500 – for *n*-pentane, 600 – for *n*-hexane, 700 – for *n*-heptane, and so on, that is, $KI = (100 \times n)$, for any *n*-paraffin where *n* – is the number of carbon atoms in the hydrocarbon chain (Figure 3.11A). The KI (isothermal indices) are calculated based on the following formula:

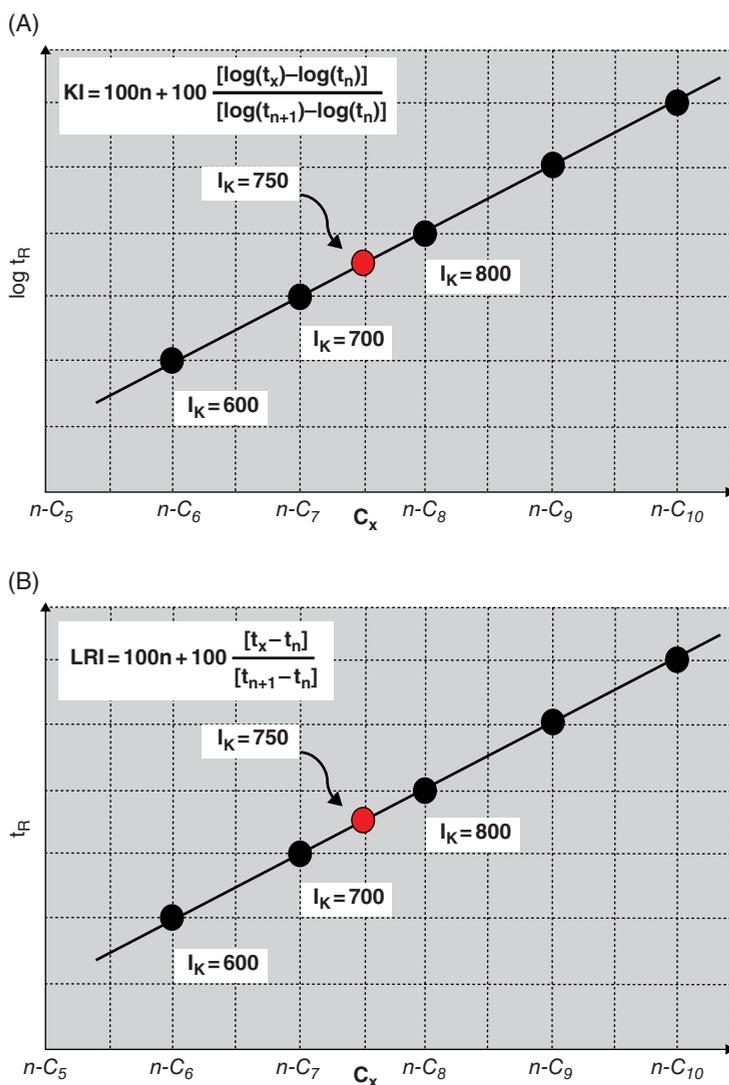


Figure 3.11 **A.** Kovats retention indices (KI) calculation, when the regime of the chromatographic oven is isothermal. The logarithm of the n -paraffin retention time depends on the number of carbon atoms. **B.** Linear retention indices (LRI) calculation when the chromatographic oven temperature is programmed. The n -paraffin retention time depends on the number of carbon atoms.

$$KI = 100n + 100 \frac{[\log(t_x) - \log(t_n)]}{[\log(t_{n+1}) - \log(t_n)]} \quad (3.1)$$

where n is the number of carbon atoms in the n -paraffin eluting before the compound of interest (its retention time is t_x) and t_n and t_{n+1} are the retention times of the n -paraffin with n and $n + 1$ carbon atoms, respectively, eluting immediately before and after the target analyte.

It has been hypothesised that retention indices become independent of most experimental conditions (temperatures of the injector and detector systems, carrier gas flow and, especially, the column dimensions, *etc.*); however, they remain dependent on the stationary phase polarity and to some extent, on column temperature.

The chromatographic analysis of the clear majority of samples requires programmed chromatographic oven temperature because their components have wide ranges of vapor pressures and molecular weights and would require very long experimental times to achieve acceptable resolution under an isothermal regime. Under these conditions the *n*-paraffins elute from the column in 'linear' and not 'logarithmic' mode (Figure 3.11B). This enabled researchers van den Dool and D. J. Kratz (Van den Dool & Kratz, 1963) to modify eqn. 3.1 and propose the calculation of so-called linear retention indices (LRI) or programmed-temperature retention indices (pTRI) — which is widely used today — according to eqn. 3.2:

$$\text{LRI} = 100n + 100(t_x - t_n) / (t_{n+1} - t_n) \quad (3.2)$$

where *n* and *x* have, the same meaning used in eqn. 3.1.

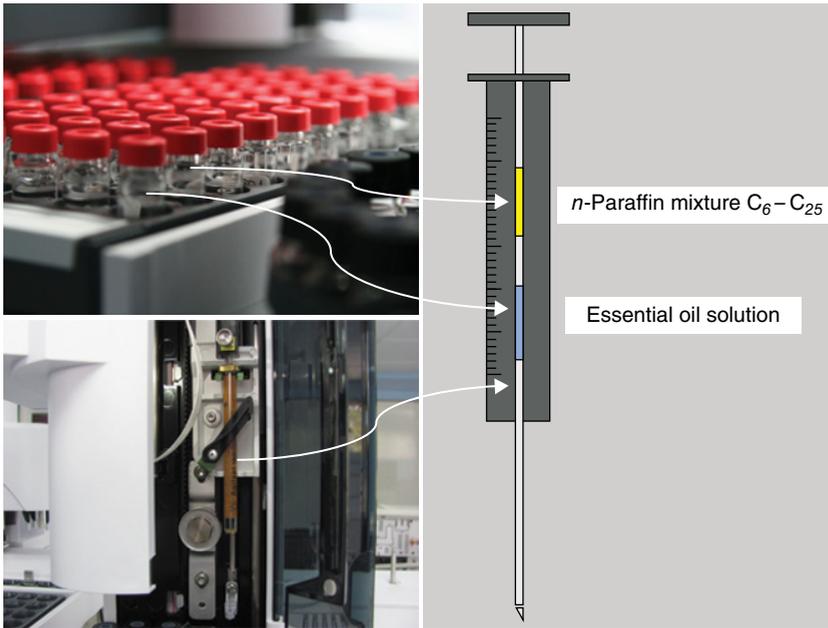
Isothermal (KI) and linear (LRI) retention indices are not identical, although they have rather close values. Then, when reporting retention indices, it is necessary to differentiate them and to indicate how they were calculated. One of the experimental approaches is to co-inject the EO sample and an *n*-paraffin mixture. The resulting chromatographic profile contains the retention times required to apply eqns. 3.1 or 3.2, depending on the oven temperature programme used (Figure 3.12).

In many articles, indices are called simply 'Kovàts indices' when, in fact, they were determined in capillary columns with temperature programming. The LRIs will eventually depend on both the initial temperature of the column, and the rate at which it increased, the stationary phase polarity and their state (contamination, ageing, presence of active sites, manufacturer). Therefore, appreciable dispersion of retention index values can be found in the scientific literature (Babushok, 2015), for the same compound and for the same stationary phase polarity, what reduces, in part, the reliability of its single use for positive identification of compounds without their mass spectra or use of certified standard substances.

The standardisation of the chromatographic conditions to determine retention indices undoubtedly leads to their greater reproducibility and confidence in their use as identification tool. However, two different substances can have the same retention index in each column. Therefore, another 'coordinate' is required, that is, to find the index in other column, with stationary phase of different polarity (polar versus nonpolar).

Nevertheless, achieving uniqueness still requires additional information, a 'fingerprint', which is a mass spectrum of the substance. In the analysis of mixtures by GC-MS, the experimental spectrum is compared with that from a database or with the mass spectrum of the certified standard substance, when it is available. In the first case, the identification would have a tentative (presumptive) character and lead to structural recognition; in the second case, when a standard compound is used, the identification of the analyte is considered positive (confirmatory), which is, when the three parameters, LRI in two columns with polar and non-polar phases, and mass spectra are identical.

(A)



(B)

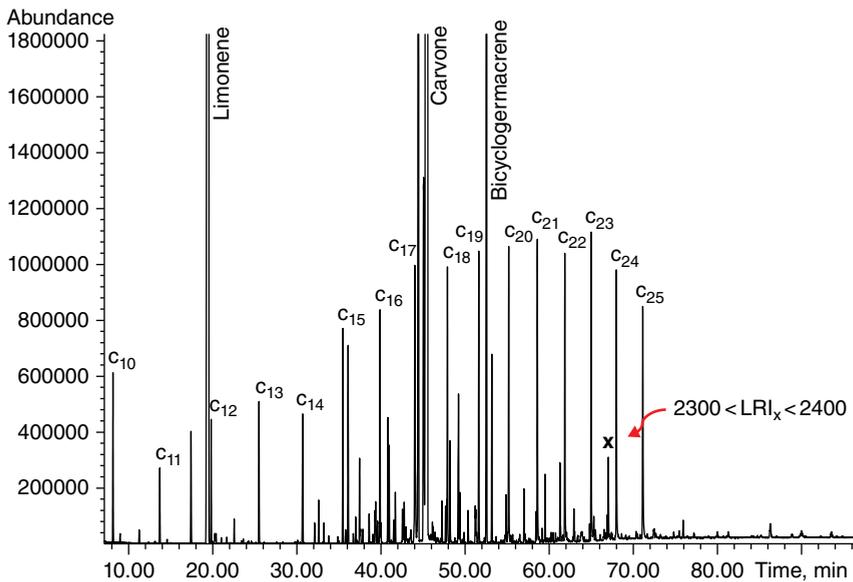


Figure 3.12 **A.** Sandwich-injection mode of an *n*-paraffin mixture and the *Lippia alba* essential oil solutions. **B.** Typical gas chromatogram used for calculation of the essential oil component linear retention indices (LRI): compound X, which elutes after C₂₃-paraffin, but before C₂₄-paraffin, will have its LRI higher than 2300, but lower than 2400 according to eqn. 3.2.

The dispersion of the values of retention indices in databases or bibliographical references, in some works found in internet portals, as well as in the values obtained by the same laboratories in particular, are due to several factors; amongst them, are the following: (1) Column: although the stationary phases may be chemically the same, there may be variations from one manufacturer to another, in their activity and retention capacity; (2) Long-term use and possible contamination and activation of the column can also influence retention index variation; (3) The measurement at different temperatures or heating rates of the column and their calculations without considering the 'isothermal' or temperature-programmed intervals may lead to not identical index values for the same compound; and finally, (4) The errors in the assignment of structural compounds, which lead to confusion when using the retention index values as the only criteria for identifying substances. An excellent review of different historical aspects and basic concepts related to retention indices, RI, their reproducibility and application, was published by Zellner and coworkers (Zellner *et al.*, 2008). They analysed and compiled the fundamental concepts and existing theories on chromatographic retention of analytes and the feasibility of comparison when they are obtained in different chromatographic systems. Alternative systems to the series of *n*-alkanes as reference substances were discussed to determine retention indices. Very useful and practical recommendations were made for RI use in the analysis of complex mixtures.

Because of its great practical utility, the work of Babushok and Zenkevich (Babushok & Zenkevich, 2009; Babushok *et al.*, 2011) deserves special mention. It is a statistical analysis of the RI of the most frequently reported compounds in different EOs and fragrances, based on information gathered from some of the available databases, books, articles and reliable internet portals, which contain information about retention indices of different analytes (terpenes, fragrances, semio-chemicals, pheromones, drugs, substances of environmental interest, *etc.*). Retention indices obtained in three commonly used columns with stationary phases 100% – poly(dimethylsiloxane), 5% – phenyl-poly (methyl siloxane) and poly (ethylene glycol) are compared. These authors emphasised that, in general, the retention index values measured on non-polar columns (OV-101, HP-1, DB-1, SE-30, Ultra-1, *etc.*) are more reproducible (± 1 –10 units) than those determined in polar columns (Carbowax 20M, Innowax, CP-WAX 52 CB, DB-WAX, *etc.*). Polar columns, with poly (ethylene glycol) (PEG) as stationary phase have higher RI value dispersions (± 10 –50) because the reproducibility of their properties and chemical characteristics is lower than that of poly(dimethylsiloxane), due to wide range of average molecular weights of PEGs, different level of cross-linking and treatments received, amongst other variables. Greater RI variability is observed when the analyte and stationary phase are of different polarity, for example, eugenol on dimethylsiloxane, pinene on PEG. However, a very large deviation of the retention index of a given analyte when compared to those reported in the literature or a database (Adams, 2007; Babushok & Zenkevich, 2009) implies, in most cases, that the compound 'identification' was not correct.

3.3.3 Mass Spectrometry

Mass spectrometry is one of the techniques used, together with other instrumental methods (UV-Vis, IR, NMR, x-ray, *etc.*), to establish the chemical structure, molecular

mass, and elemental composition of molecules, mostly organic or bioorganic (biopolymers). All modern MS techniques can be divided roughly into two groups, depending on the chemical nature and the molecular mass of the analysed molecule as follows: (1) MS of volatile molecules with low- to medium-molecular masses (up to 450 Da), thermostable and moderately polar, and (2) MS of non-volatile, thermolabile polar molecules or macromolecules (proteins, polysaccharides). EO components correspond to the first group. Their separation is performed with gas chromatography, coupled to various possible mass spectrometric detection systems (Marriott *et al.*, 2001).

Despite the utmost importance of macromolecules (in the age of genomics and proteomics) and very important developments in MS techniques for their analysis, as well as for very polar or non-volatile molecular species, a constant interest is held and focused on molecules with relatively low- or medium-molecular masses, thermostable, medium- or highly volatile substances. They include environmental contaminants, quality control substances for food and products often used by mankind (flavours, fragrances, additives, antioxidants, toiletries, *etc.*), pheromones, plant volatiles, allelochemicals, pesticides, secondary metabolites from plants, explosives, arson accelerators, many drugs and their metabolites and thousands of other molecules of scientific, regulatory, commercial or technical interest. Many of these substances are found within complex mixtures (gasoline, perfume, a plant extract, vegetable or fruit flavours, coffee, cocoa, chips, volatile organic compounds in air or water, pesticides, *etc.*), many at trace level (ppb, ppt), and it is impossible to obtain information about their identity (*in situ*, directly in the mixture) by methods other than gas chromatography (GC) coupled with mass spectrometry (Hübschmann, 2009).

3.3.3.1 Ionisation Processes

Mass spectrometric analysis requires the ionisation of the molecule. This may be followed by chemical bond cleavage. The mechanisms that provide the energy for molecular ionisation include collisions with photons, with accelerated atoms, ions or electrons, or the exposition to a high electrostatic field gradient, or thermal impact. Electron ionisation (EI) is the oldest and most widespread technique for organic molecule ionisation. An electron that belongs to the molecule interacts with the bombarding electron and energy is acquired by the molecule. Ionisation takes place and the remaining excess energy originates scissions that give rise to the fragment-ions that are detected by the spectrometer. Chemical ionisation (CI) is a soft ionisation method which is used as alternative approach, in the case of very labile molecules whose mass spectra contain no molecular ion signals when they are obtained by EI (Ashcroft, 1997). Depending on the analyte's chemical nature and the reagent gas used for CI, either positive or negative ions are formed. Protonation (MH^+), deprotonation ($M-H^-$) or electron capture (M^-) may take place in CI. The analyte's molecular weight is readily determined from the signal of the resulting modified molecular ion.

Gas chromatography-mass spectrometry uses both electron ionisation (EI) and chemical ionisation (CI). Since CI requires much higher pressures (up to 1 Torr) than EI (10^{-5} – 10^{-7} Torr), the volume of the ionisation chamber should be changed when it is modified from one to the other ionisation technique. The application of EI or CI is limited by the volatility or thermal instability of many organic substances. It is estimated that less than 10% of all organic substances can be ionised in the gas phase by either one of these techniques.

In EI or CI, volatilisation and ionisation are separate events in time and space. Techniques for substances of low volatility, such as matrix-assisted laser desorption (MALDI), combine the two steps in a same process. However, the EO analytes are volatile and amenable to EI or CI. Once the ions are formed in the ionisation chamber, coulombic forces are used to control their movements. Metallic electrodes with positive or negative potentials permit to collimate (focus) and accelerate the ions and direct them to a mass analyser. The ions are then separated per their m/z ratio. Quadrupole (Q) and ion trap (IT) are the most common mass analysers used in GC-MS instruments. The need for higher resolution has been a powerful driving force in recent years for the increase in the use of time-of-flight analysers (TOF), resonance ion-cyclotron analysers with Fourier transform (FT-ICR) and the Orbitrap, which has been commercially available for more than a decade and is now being used with GC. The use of magnetic deflection mass analysers is nowadays much less frequent (Xian *et al.*, 2012).

3.3.3.2 Ion Types and Fragmentation Patterns

In most cases, dissociation follows molecular ionisation. The excess energy may be employed in protonation or deprotonation processes, electron subtraction or addition, nucleophilic or electrophilic routes, and cluster formation, to produce ions. Collisions are very infrequent because of the low pressure of the ionisation chamber (10^{-5} – 10^{-7} Torr, mean free path of several metres). Therefore, these are endothermic monomolecular dissociation processes that result in M^{+} fragment ions.

Simple (homolytic or heterolytic) rupture reactions and molecular rearrangements are the two types of dissociative ionisation processes. The rearrangements may consist of a simple hydrogen transposition, as in the case of the well-known 'McLafferty rearrangement', or they may involve a whole molecular skeleton rearrangement, as when the tropylium ion, $C_7H_7^+$, at m/z 91 is formed from the ionisation of a benzyl fragment (McLafferty & Tureček, 1993). The formation of cations from hydrocarbons, the allylic and benzylic ruptures, acyl formation and retro Diels-Alder reactions (RDA) in cyclic monounsaturated systems, are the most common simple rupture reactions. As a rule, less energy is required for rearrangements than for simple ruptures. The latter processes are in general less vulnerable to steric effects and are less selective than hydrogen or skeleton transpositions.

The comparison of mass spectra obtained from the same substance at 70 eV or at low voltage (10–30 eV) reveals strong differences (Figure 3.13). The relative intensity of molecular ions prevails (higher relative abundance) in the latter. The M^{+} dissociation is accompanied by a few products, basically, from molecular rearrangements and hydrogen transpositions, since they require lower activation energy.

The fragmentation process is a very distinctive series of events for each substance. Bond ruptures and molecular rearrangements do not happen at random. What fragment ions result from the process and their abundance (fragmentation pattern), are a direct consequence of the molecular structure, the bombarding electron energy and how much internal energy remained in the excited ionised molecule. The system pressure, the contamination of the ionisation chamber, or the deterioration of the electron multiplier that acts as final fragment ion-electric signal transducer, affect the ionic current, or number of ions detected, but not the fragmentation pattern. The latter and the ion types produced, depend on molecular structure, on the excess energy which the molecule acquires during ionisation, on the activation energies of possible processes and

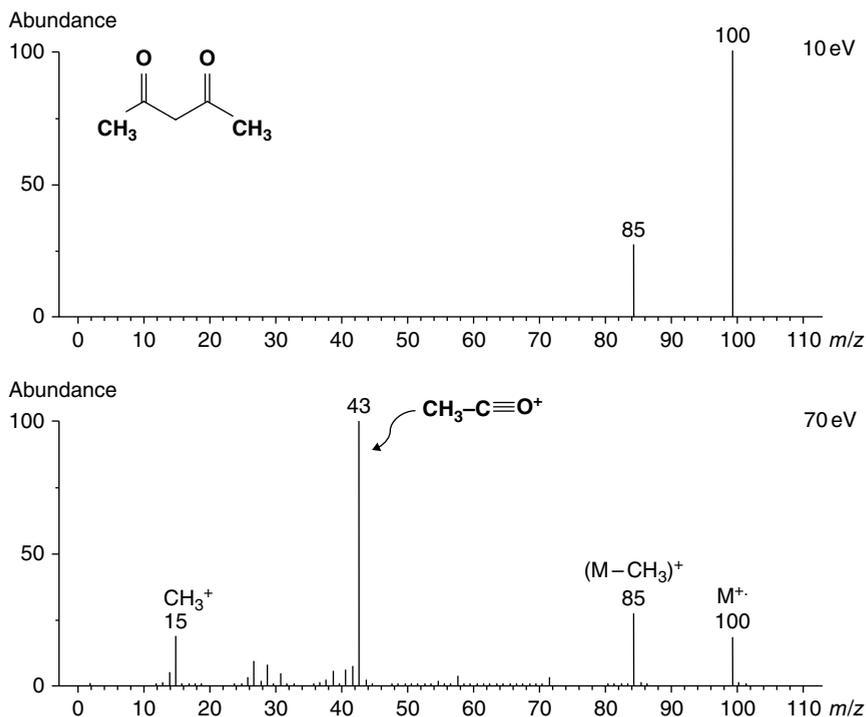


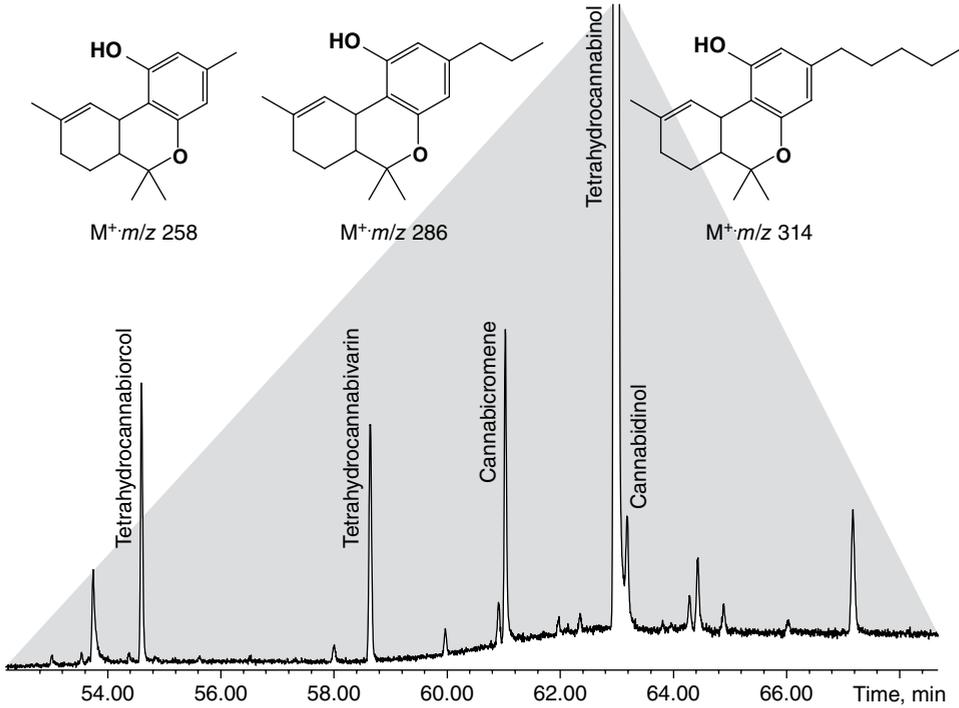
Figure 3.13 Mass spectra and typical fragment-ions of 2,4-diketopentane, obtained at different electron-ionisation energies (10 eV and 70 eV).

their dissociation rate factors (bond cleavage). The methods by which they can be used to elucidate a molecular structure are discussed in the next section.

3.3.3.3 Mass Spectra Interpretation

During the interpretation of the results obtained by GC-MS, the need to decipher, with high degree of reliability — and establish the molecular structures of substances in complex mixtures, arises because certified standard substances are not always available for all the analytes. It is interesting to study the fragmentation pattern of homologous or similar substances to apply this knowledge to new, similar structures (Figure 3.14). Thus, structural assignment cannot be limited to comparing retention indices and mass spectra with those of the certified standards. It is often impossible to recognise the chemical structures only by comparing their mass spectra with those of commercially available databases (NIST, WILEY, specialised libraries), because these do not have all the compounds which can possibly exist in a real mixture. The structures of the cannabinoids tetrahydrocannabinol, tetrahydrocannabivarin and tetrahydrocannabinol differ only in the length of the side chain at position 3 (1, 3, and 5 carbon atoms, respectively). In these cases, a ‘manual’ interpretation (Figure 3.14, Table 3.1) of the experimental mass spectra could be done. This means analysing fragmentation patterns or dissociation reactions of molecules ionised by EI and their ion-fragments, to obtain a good representation of the original molecular structure. The mass spectra of these three cannabinoids are very similar, but their comparison reveals a difference of 28 units for

(A)



(B)

Scan 15114 ($t_R=54.59$ min)

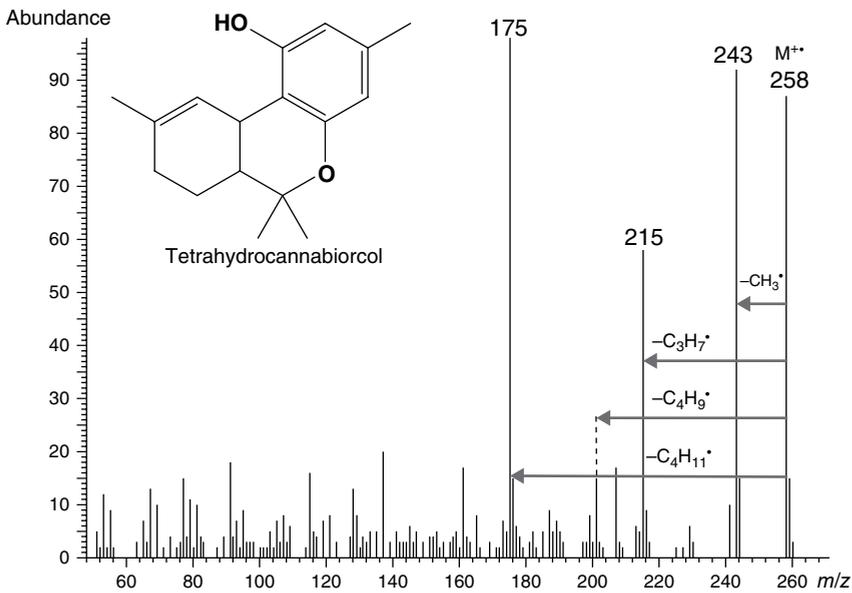
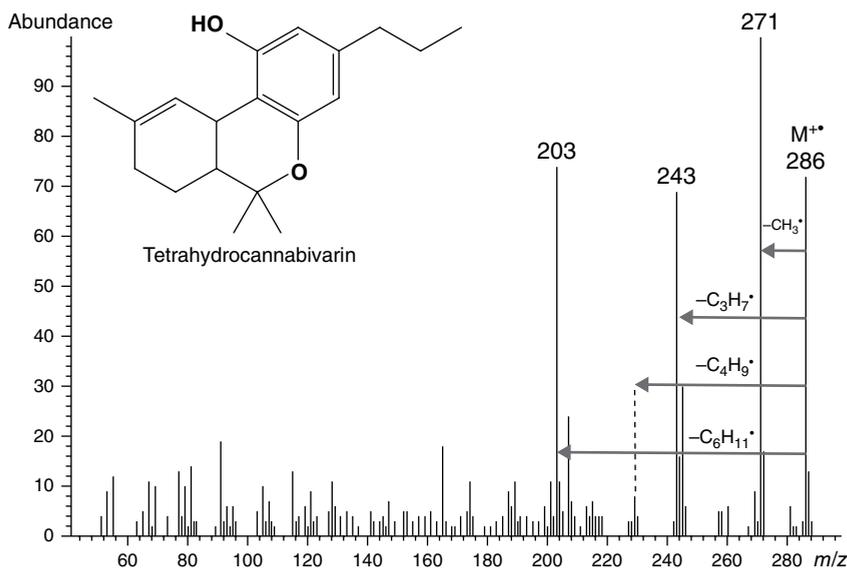


Figure 3.14 GC-MS analysis of the essential oil isolated by hydrodistillation from *Cannabis sativa* inflorescences. **A.** Part of the chromatogram (TIC, GC-MS, see **Figure 3.5B**) of hydrodistilled *C. sativa* in florescence essential oil: different GC peaks correspond to various cannabinoid compounds, and their mass spectra (EI, 70 eV, m/z 50–350), as follows: **B.** Tetrahydrocannabinol, C_{21} ; **C.** Tetrahydrocannabivarin, C_{19} , and **D.** Tetrahydrocannabinol, C_{21} . Similar fragmentation patterns are observed for these three homologous compounds (Table 3.1).

(C)

Scan 15114 ($t_R = 58.63$ min)

(D)

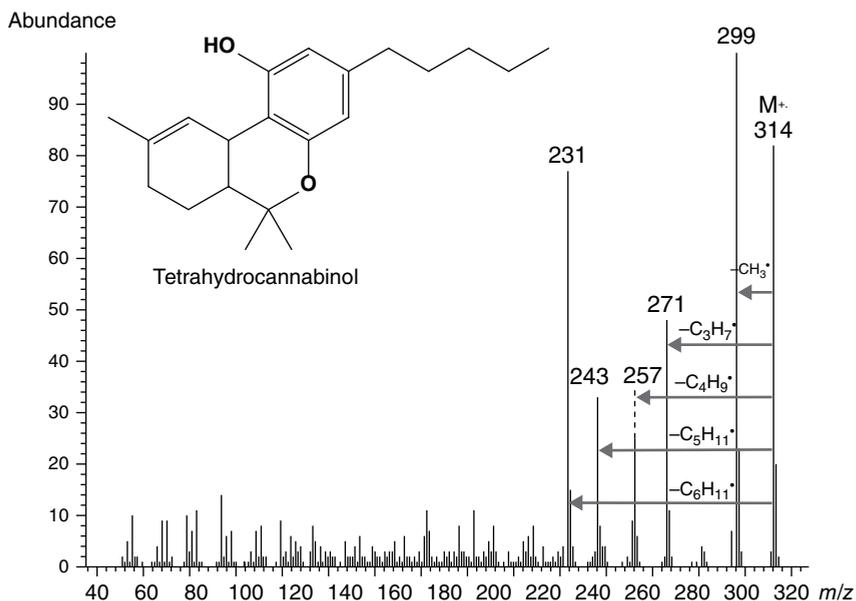
Scan 15114 ($t_R = 62.98$ min)

Figure 3.14 (Continued)

several groups of signals, when changing from one compound to the next. The tetrahydrocannabinol mass spectrum is not found in spectral databases (*e.g.*, NIST 14), but it can be identified, because its fragmentation pattern is like that of the other two cannabinoids.

Table 3.1 Molecular ion (M^+) and some typical fragment-ions (m/z , intensity, %) in the mass spectra (EI, 70 eV) of three homologous cannabinoids.

Compound	m/z (Intensity, %, n = 3)					
	M^+	[M - CH ₃] ⁺	[M - C ₃ H ₇] ⁺	[M - C ₄ H ₉] ⁺	[M - C ₅ H ₁₁] ⁺	[M - C ₆ H ₁₃] ⁺
Tetrahydrocannabinol	258 (90–93%)	243 (97–100%)	215 (58–62%)	201 (10–15%)	187 (2–5%)	175 (97–100%)
Tetrahydrocannabivarin	286 (75–78%)	271 (100%)	243 (70–74%)	229 (8–10%)	215 (3–5%)	203 (75–78%)
Tetrahydrocannabinol	314 (85–87%)	299 (100%)	271 (50–55%)	257 (32–35%)	243 (35–38%)	231 (79–81%)

The appearance of a mass spectrum (partial ion current, *i.e.*, intensity) of an organic molecule, obtained by EI, depends on several factors: (1) Chemical nature of the molecule (ionisation potential, cross section); (2) Energy of bombarding electrons; (3) Ion flight time from the ionisation chamber, through the analyser towards the detector; (4) Residual pressure of the instrument; and, crucially, (5) Internal ion energy, bond strength and the activation energy required to form a production. Obviously, the intrinsic, qualitative factors belonging to an organic molecule, that determine its mass spectrum, are its chemical structure and the internal energy (excitation energy) it acquires during ionisation.

Thymol and carvacrol are phenolic isomers whose structural difference is the hydroxyl group position. Their mass spectra and those of their acetates are remarkably similar (Figure 3.15), but their retention indices differ sufficiently to permit their distinction (Table 3.2).

The mass spectrum provides information on the particular m/z ions detected after an ionised molecule dissociates. This fragmentation pattern (m/z values and their intensity) depends on which atoms are bonded, the strength of these chemical bonds, their spatial disposition, the molecule's ionisation potential and the internal energy that it acquired during an electron collision. This pattern is unique to each molecular structure and contains unmistakable differences (often just quantitative), even for isomers. Figure 3.16 contains mass spectra of the four isomers, *cis*- and *trans*-hexatriene, 1,3-cyclohexadiene and 1,4-cyclohexadiene. Despite a marked qualitative similarity of their spectra, there are some distinguishable quantitative differences in the intensity of the molecular ions and the relative intensities of the fragment ions. These fragmentation patterns are very similar due to their molecular ion rearrangement to a structure common to all four isomers. There is a gradual loss of hydrogen atoms from the molecular ions of all isomers and the occurrence of fragments at m/z 79, 78, 77, which illustrate a trend towards aromatisation (stabilisation) in both cycles, and polyunsaturated ionised molecules, which can be rearranged in cycles, after ionisation.

Mass spectra of different molecules may contain common fragments due to the presence of the same functional group in their structures, as illustrated by the dihydroeudesmol and α -terpineol mass spectra (Figure 3.17).

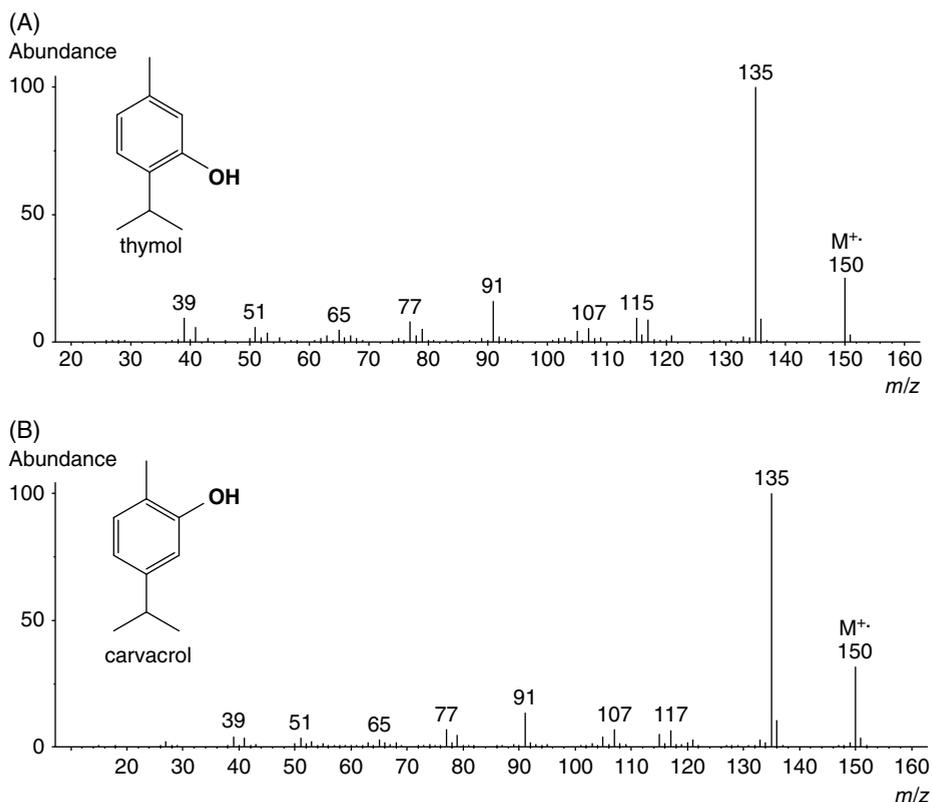


Figure 3.15 Mass spectra (electron ionisation, 70 eV) of isomeric phenolic compounds, present in many essential oils (e.g., thyme, oregano, *Lippia origanoides*, *L. micromera*): **A.** Thymol. **B.** Carvacrol and their acetates: **C.** Thymyl acetate and **D.** Carvacryl acetate. Although the mass spectra of these isomeric compounds are similar, their linear retention indices (LRI) vary in the columns of different stationary phase polarities (Table 3.2).

Mass spectra obtained by EI register charged species with lifetime longer than μs . These are typically molecular ions, fragment-ions and isotopic ions. Sometimes there are signals from multicharged ions (formed from polyaromatic compounds, for example). Metastable ions, m^* , which are products of ion fragmentation outside the ionisation chamber, may be detected in mass spectrometers with specific configurations (mostly with magnetic field deflection). For the structure elucidation of the molecule, each of these ions provides structural information of greater or lesser importance.

Table 3.3 presents a summary on the ion types that are recorded in EI mass spectra and the structural information they may provide. At present time, the tandem MS configuration can replace the use of the m^* ion to establish the fragmentation pattern (Section 3.4.2).

Fragment ions can be products of both monomolecular single rupture, and rearrangement (skeleton, hydrogen transpositions) reactions. While single rupture reactions are generally characterised by their very high reaction rate, rearrangement processes are slower, but could have lower activation energies. The peaks of low-voltage

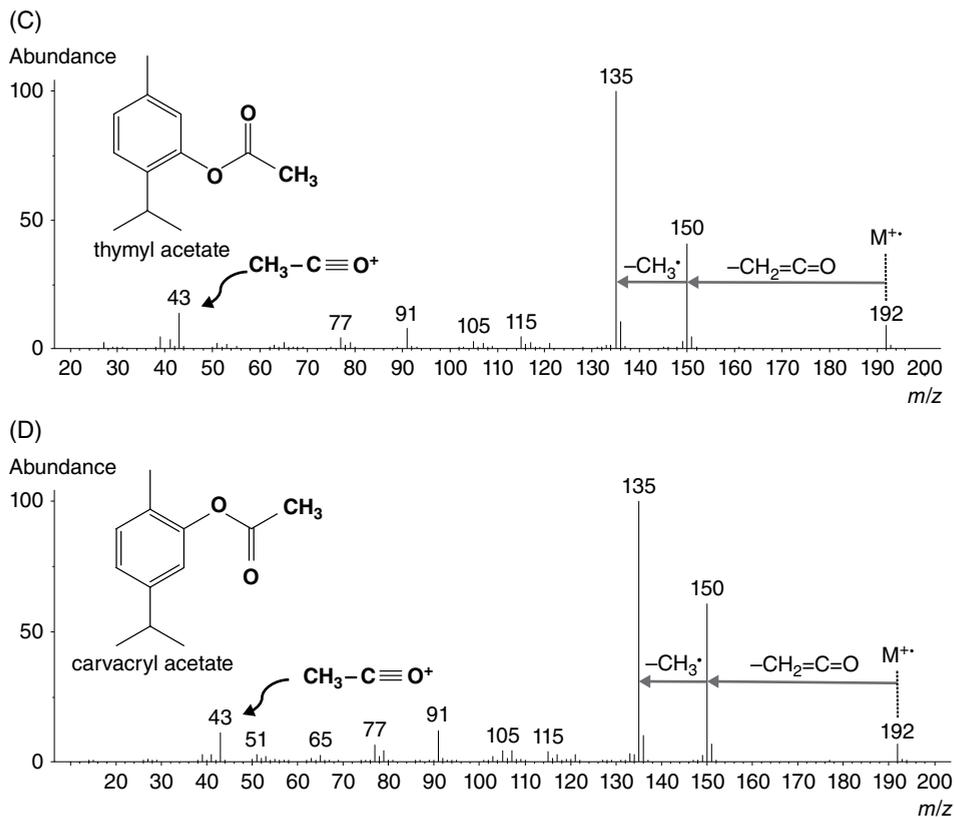


Figure 3.15 (Continued)

Table 3.2 Linear retention indices (LRI) of isomeric essential oil phenolic compounds (thymol, carvacrol and their acetates), calculated in columns with different stationary phase polarities.

Compound	Formula	Retention indices, average value (90% RI range)		
		PDMS (DB-1, HP-1)	5%-Ph-PDMS (DB-5, HP-5)	PEG (DB-WAX)
Thymol	$\text{C}_{10}\text{H}_{14}\text{O}$	1272 (1260–1289)	1290 (1272–1304)	2164 (2100–2205)
Thymyl acetate	$\text{C}_{11}\text{H}_{16}\text{O}_2$	1343 (1330–1351)	1356 (1350–1364)	1867 (1783–1945)
Carvacrol	$\text{C}_{10}\text{H}_{14}\text{O}$	1283 (1272–1300)	1300 (1291–1314)	2211 (2140–2246)
Carvacryl acetate	$\text{C}_{11}\text{H}_{16}\text{O}_2$	1354 (1344–1367)	1373 (1364–1391)	1880 (1868–1890)

From Babushok, V.I., P.J. Linstrom, P.J., & Zenkevich, I.G. (2011) Retention indices for frequently reported compounds of plant essential oils. *J Phys Chem Ref Data*, 40(4): 1–47.

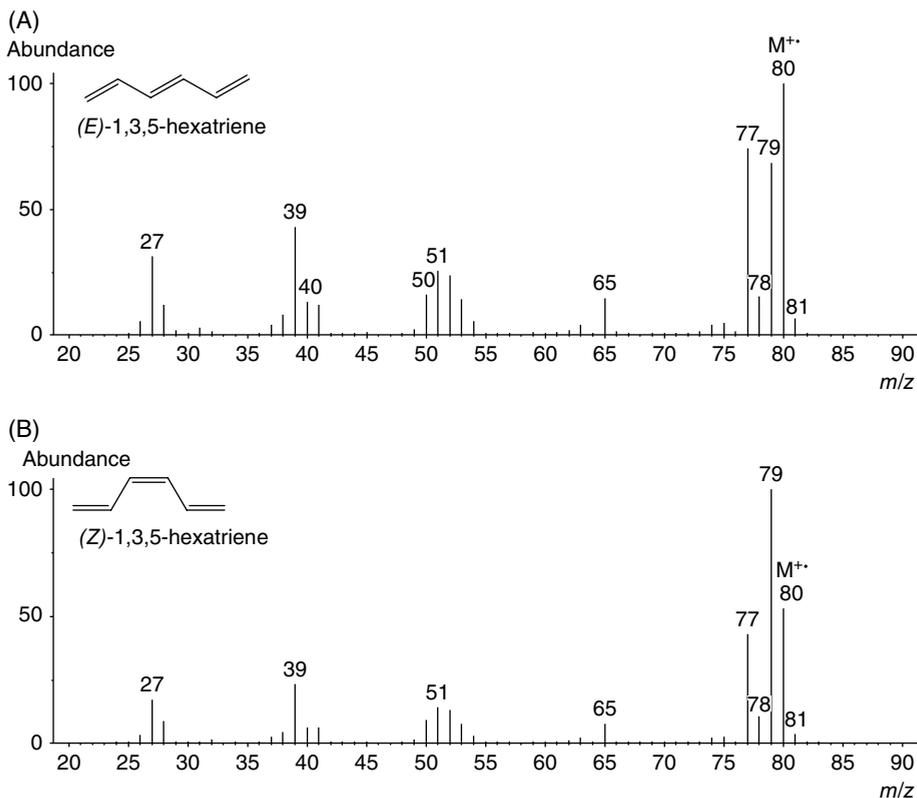


Figure 3.16 Mass spectra (electron ionisation, 70 eV) of isomeric hydrocarbons: **A.** (*E*)-1,3,5-Hexatriene; **B.** (*Z*)-1,3,5-Hexatriene; **C.** 1,3-Cyclohexadiene and **D.** 1,4-Cyclohexadiene. The high similarity of these mass spectra could be explained by the formation of the molecular ion, $M^{+\bullet}$, rearranged in the same molecular structure, during the ionisation process of these isomers.

mass spectra may correspond mainly to rearrangement products and meta stable ions. The fragmentation processes of both single rupture and rearrangement are varied; however, usually they turn out to be common (α - and β -breaks, *ortho* effect, McLafferty rearrangement) for molecules belonging to different families of compounds; some of these frequently monomolecular reactions in the mass spectra of many organic compounds, are summarised in Table 3.4.

The study of dissociative ionisation of substances with low- or medium-molar mass is based not only on theoretical assumptions, for example, on qualitative theories about the stability of fragmentations and neutral particles or on the theory of positive charge or radical centre location in an ionised molecule, or on general rules derived from organic physical chemistry, but first, is based on experimental results, that is, on mass spectra. There is a wide array of experimental methods to establish the fragmentation path of an ionised molecule, but, unfortunately, not all of them can be executed in the same mass spectrometer because they require several techniques, tests and approaches; some of them are purely chemical strategies (derivatisation), some are instrumental (photoionisation, activated collisions, bimolecular reactions, tandem configurations, *etc.*) and some other are theoretical methods and quantum mechanical calculations.

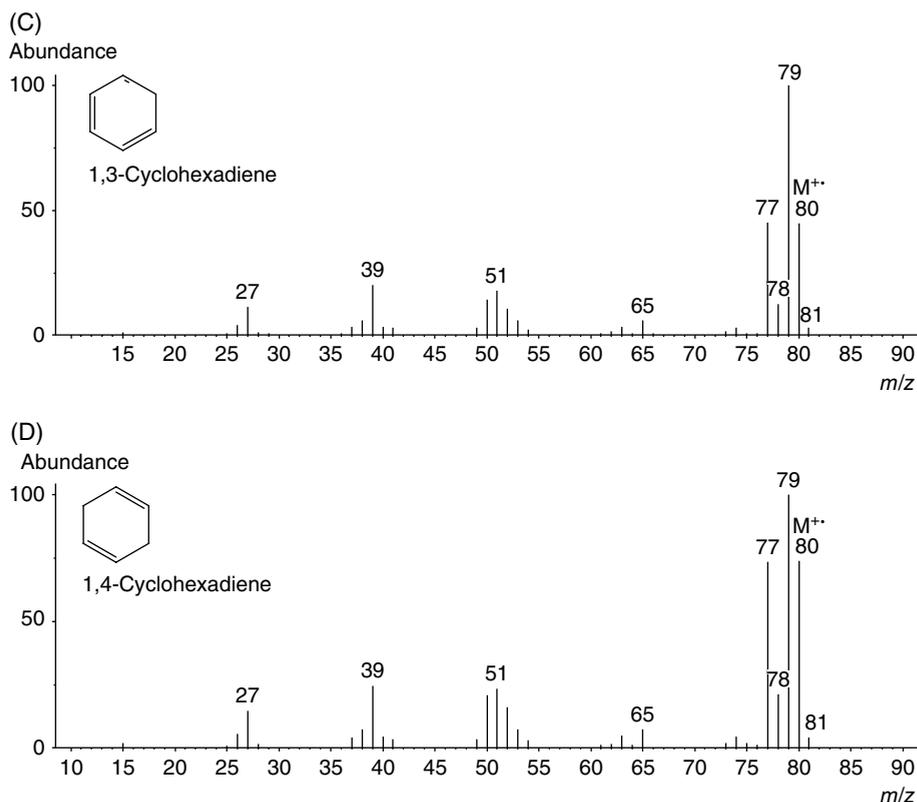


Figure 3.16 (Continued)

Each molecular ion is characterised by two fundamental properties: structure and reactivity. Only in the gaseous phase, at high vacuum, the intrinsic or own properties of molecules or ions become apparent, as there is no influence of other molecules, and the association effects (typical for the condensed phase) are absent. There is no energy exchange, a thermodynamic equilibrium is not achieved in the system; molecular species in a vacuum show their intrinsic reactivity, clearly derived from their structure and from the energy acquired during ionisation. They are in equilibrium with themselves. The ions formed in the mass spectrometer can be divided roughly into two groups: (1) those that do not dissociate and are stable (life time over 10^{-4} – 10^{-5} s) and (2) those that suffer fragmentation in the ionisation chamber (fragment ion) or outside it (metastable) before detection. The establishment of the structure of each of these ions, the mechanisms of their formation or fragmentation, is not a trivial task but is not impossible, although several experimental and theoretical methods should be combined.

Techniques for establishing the ionic structure or fragmentation path of an ionised molecule include, amongst others, the following: (1) isotopic (^2D , ^{18}O , ^{15}N) and chemical (introduction of groups, *e.g.*, $-\text{F}$, $-\text{OCH}_3$) labelling; (2) determination of exact masses of ions and the study of isotopic distribution; (3) study of metastable transitions or use of modern tandem techniques; (4) combination and comparative study of electron

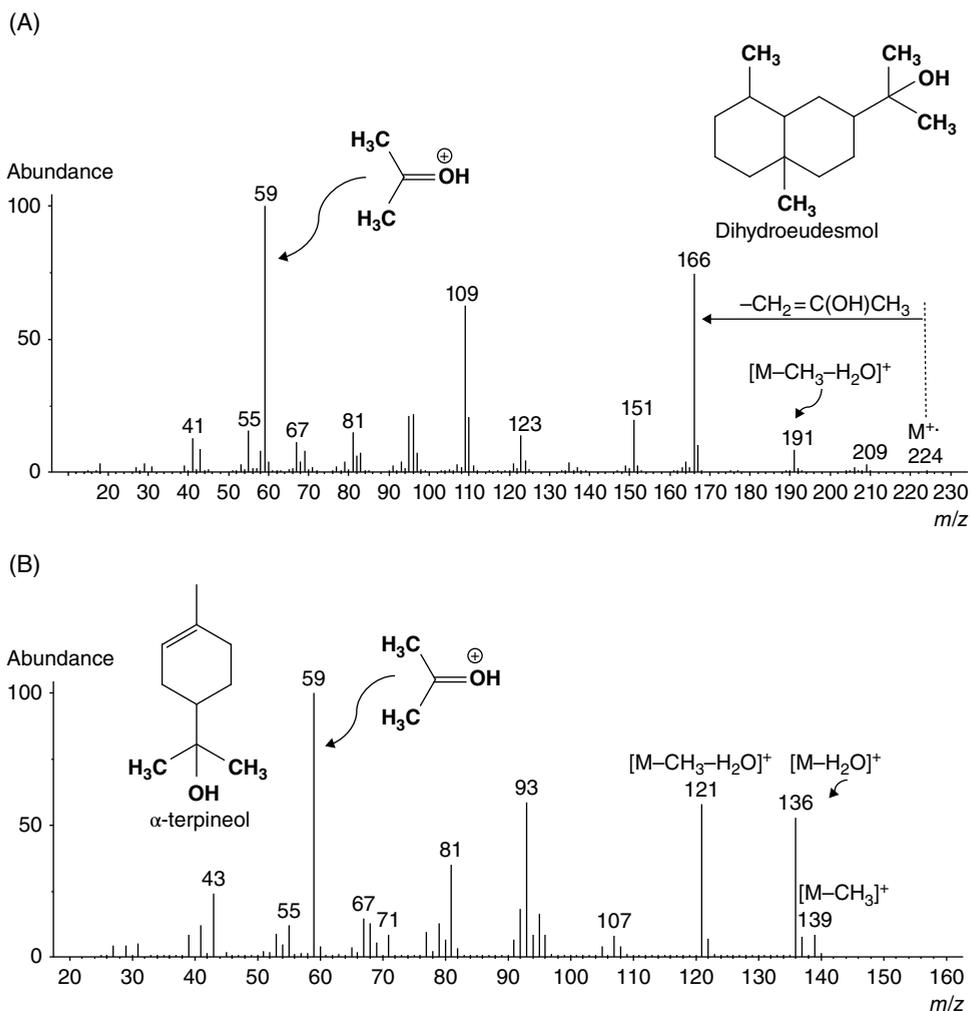


Figure 3.17 Mass spectra (electron ionisation, 70 eV) of two terpenic compounds: **A.** Dihydroeudesmol; **B.** α -Terpineol. Formation of the common fragment-ion at m/z 59.

spectra obtained with different energies or different ionisation methods (*e.g.*, EI Vs CI); determining electronic or proton affinities; study of bimolecular reactions; (5) determination of ionisation energies of molecules and appearance potential of fragment ions; (6) determination of chemical bond energies, activation energies of dissociation processes or other molecular thermodynamic parameters; photoionisation spectra study; (7) study of dissociation reactions induced by activated ion collisions or photo-dissociation; (8) quantum mechanical calculations of the total energies of formation or ionic structures and the structure elucidation of most probable fragments (Hübschmann, 2009; Kandiah & Urban, 2013; McLafferty & Tureček, 1993). The results of all these methods cannot always be complemented, although most of these are indeed essential to correctly set the path and, even more, the mechanism of fragmentation of an ionised molecule.

Table 3.3 Types of ions in mass spectra obtained by electron ionisation and the structural information that they may provide.

Information	Examples
<p>Molecular ions</p> <p>Molecular mass (elemental composition when high resolution MS is used). High intensity molecular ion signals indicate structural capacity to stabilise positive charge, as for example, in aromatic molecules. When the molecular ion signal is not registered in the spectrum, a soft ionisation technique, such as CI, should be used. Odd mass implies an odd number of nitrogen atoms in the molecule.</p>	<p>The stability ($W_M, \%$) of the molecular ion is the percent fraction of the total ion current obtained after the ionisation and fragmentation of a substance. The values of W_M for hexane (2.8%), cyclohexane (4.6%), 1,3-cyclohexadiene (16.5%) and benzene (33.2%) show its strong dependence on the charge stabilisation capacity of the molecular structure.</p>
<p>Fragment ions</p> <p>The fundamental structure elucidation information is contained in their m/z values and relative abundances. These ions may be cations (even electron number), or radical-cations (odd electron number), which may result from simple rupture or from rearrangement, respectively. The loss of a fragment with a specific mass may indicate its presence in the molecule that dissociates after ionisation.</p>	<p>Molecular ions of alcohols easily lose an OH[•] group or a water molecule, generating the corresponding radical-cation ($M-17$)⁺ and ($M-18$)⁺ fragments. Acetates lose a ketene from the molecular ion and it is thus characteristic of acetates to exhibit an ($M-CH_2=C=O$)⁺ fragment in their mass spectra (Figures 3.15C and 3.15D). Fragment ions $C_6H_5^+$ and $C_7H_7^+$ dissociate with the loss of an acetylene molecule and the generation of signals at m/z 51 and 65, respectively, and may indicate the presence of a phenyl or benzyl group, respectively, in the molecule (Figure 3.18).</p>
<p>Isotopic ions</p> <p>Contribute information on the elemental composition of the substance. They allow easily discerning the presence of some heteroatoms such as halogens Cl, Br, S, or Si, to calculate the number of carbon atoms in the molecule, double bonds, <i>etc.</i> The presence of signals from isotope ions is 'mandatory' in the spectrum, as it is one of the quality criteria of a mass spectrum.</p>	<p>The successive loss of chlorine atoms from the molecular ion of polychlorinated biphenyls (PCBs) forms the respective ($M-nCl$)⁺ fragments in the mass spectrum of each one of the PCB congeners and together with the isotopic distribution pattern of the molecular ion, permit to establish the number of chlorine atoms present in the molecule.</p>
<p>Multicharged ions</p> <p>Are formed only in the mass spectra of a class of substances, for example, those containing heteroatoms (N, S, O), aromatic or heteroaromatic rings, high degree of unsaturation (conjugated bonds) or when several of these elements are combined in the molecule.</p>	<p>They have relatively low intensity. They can have sometimes fractional values. For example, the signal at m/z 64 in the mass spectra of naphthalene (M^+, m/z 128) corresponding to the doubly charged molecular ion, M^{2+}. In the mass spectrum of pyrene, the fragmentation at m/z 101 corresponds to the double-charge molecular ion.</p>
<p>Metastable ions</p> <p>These are often recorded on magnetic deflection mass spectrometers. They are formed out of the ionisation chamber, from ions having lifetime longer than 10^{-6}s, but shorter than the time needed to reach the detector without dissociating. They appear as diffuse peaks, with an apparent mass (fractional number), m^*, related to the parent ion (m_1) and daughter (m_2) masses by the equation $m^* = m_2^2/m_1$.</p>	<p>In a benzoic acid mass spectrum, metastable transitions (m^*) corresponding to processes of the OH[•] radical and the CO losses from the molecular ion, as follows: $M^+ \rightarrow (M-OH)^+$ and $(M-OH)^+ \rightarrow [(M-OH)-CO]^+$, confirming the 'genetic' link between these ions; however, a metastable transition corresponding to a possible direct loss of COOH group from M^+ as in the $M^+ \rightarrow (M-COOH)^+$ process is not registered, since no m^* ion appears to confirm it.</p>

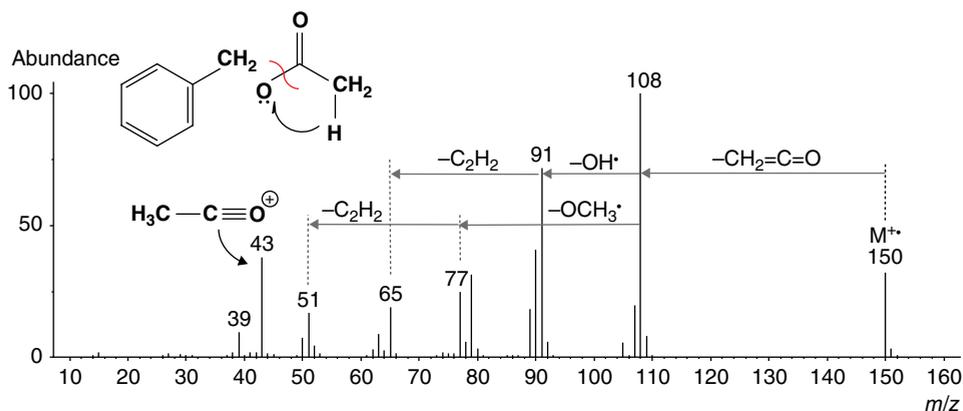


Figure 3.18 Mass spectrum (electron ionisation, 70 eV) and typical fragment-ions of benzyl acetate.

3.3.4 Hyphenated Techniques

A set of instrumental analytical methods (UV, IR, Raman, NMR, MS, *etc.*) provide information to establish the molecular structure, but their use typically requires isolation of the pure substance, which in many cases is not possible. The combination of chromatographic and spectroscopic techniques has appeared as a powerful alternative to overcome this limitation. The most widely used technique for EO analysis is precisely one of these combinations, GC-MS. The MS detection may consist of more than one analyser, as in the tandem systems to be described later. GC-MS and related techniques play a dominant role in EO analysis, but other hyphenated techniques may provide complementary information for structural characterisation.

Amongst them, LC-NMR deserves mention because of the detailed spectroscopic data that can be obtained from the isolated analytes (Kumar, 2015). Unstable substances, not amenable to the GC-MS thermal treatment, may be separated and characterised by LC-NMR. Apart from high installation and operating costs, the main limitation of this coupled technique is the low sensitivity of NMR (Marston, 2007). An interesting approach to reduce this problem is to use solid-phase extraction as an intermediate concentration step between LC and NMR, as illustrated in the work of Miliuskas *et al.* (2006).

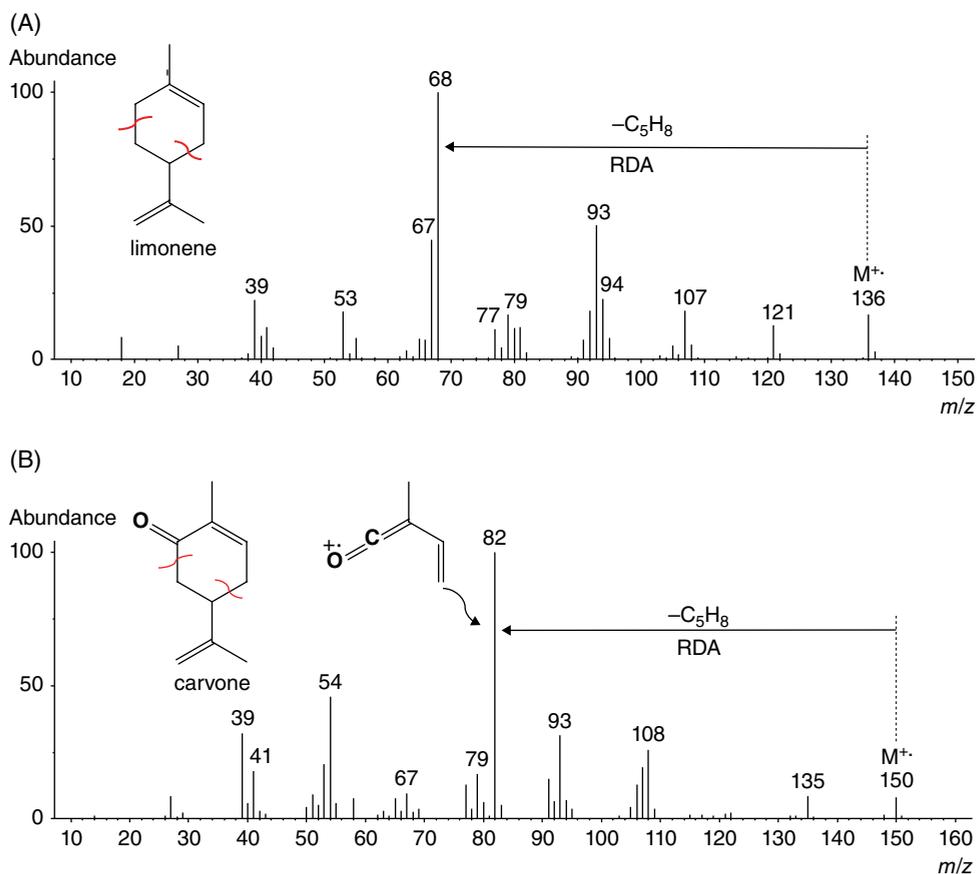
3.4 GC-MS

A mass spectrometer attached to the gas chromatograph is also often called a 'mass selective detector' (MSD). It consists of an ionisation chamber (ion source or ion volume) in which the most common method is ionisation with electrons or electron impact (EI). When no molecular ions are recorded in mass spectra obtained by EI, a complementary soft ionisation method such as chemical ionisation (CI) may be used.

In GC-MS, the chromatographic profile is the representation of the total ion current (TIC) as a function of time. The areas of the chromatographic peaks in this profile are the basis for the quantification of analytes, after calibration and determination of the response factors, which afford chromatographic corrected areas. However, the produced

Table 3.4 Examples of some of the most common dissociation processes resulting from electron ionisation (simple rupture and rearrangement) in organic molecules.

Simple rupture	Rearrangements
Cation formation Elimination of an olefin molecule from a cation Acyl-forming ions Allylic and benzylic ruptures α - and β -Ruptures Retro-Diels-Alder Rupture (RDA) (Figure 3.19)	Hydrogen transpositions McLafferty rearrangement (Figure 3.20) Elimination of a neutral molecule (H_2O , H_2S , CH_3OH , $CH_2 = C = O$, HCN , C_2H_2) Opening a cycle and hydrogen migration <i>Ortho</i> -effect (Figure 3.21) Molecular skeleton rearrangement

**Figure 3.19** Mass spectra (electron ionisation, 70 eV) of monoterpenoids: **A.** Limonene, and **B.** Carvone. Fragment-ions at m/z 68 and at m/z 82 correspond to retro-Diels-Alder reaction (RDA) products occurred through the loss of C_5H_8 from limonene and carvone molecular ions, M^+ , respectively.

ionic current strength may vary even in the same equipment, because it depends, amongst other factors, on the number of ions produced, the pressure of the ionisation chamber, the degree of its contamination, and the stability and sensitivity of the electron multiplier measurement. The pressure in the ion source, in turn, is variable, since

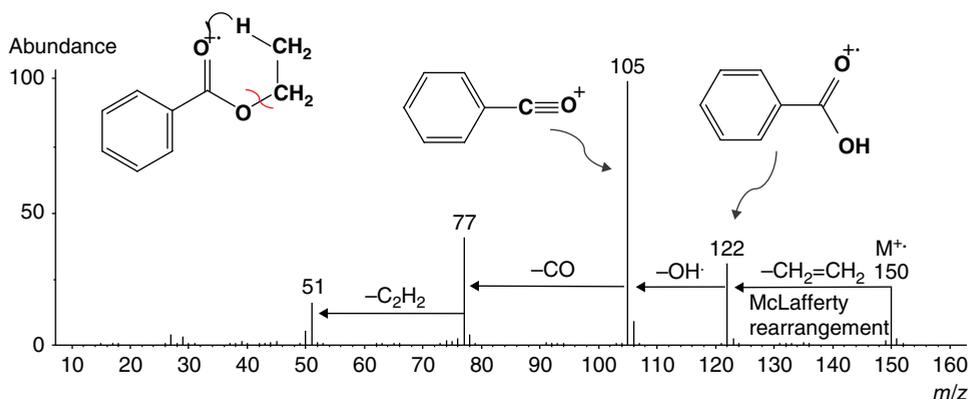


Figure 3.20 Mass spectrum (electron ionisation, 70 eV) and typical fragment-ions of ethyl benzoate. Fragment-ion at m/z 122 is a product of McLafferty rearrangement of the molecular ion M^+ . The ion at m/z 105, usually of high intensity (70–100%), is a typical fragment of benzoates.

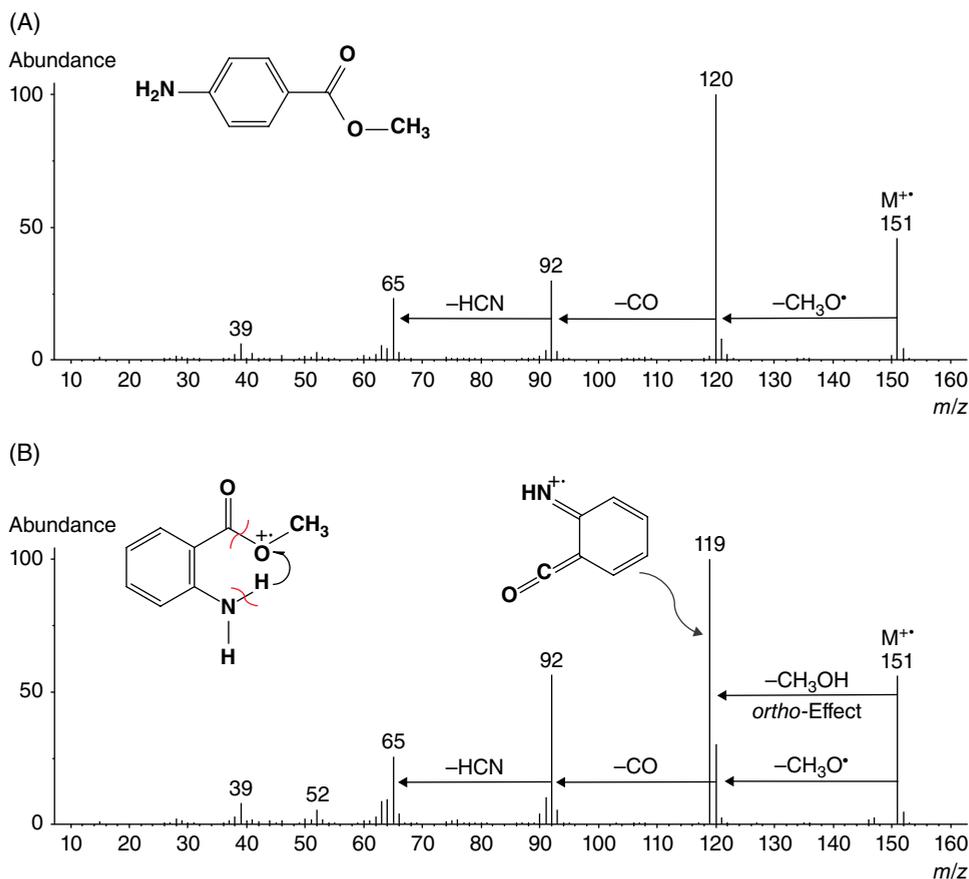
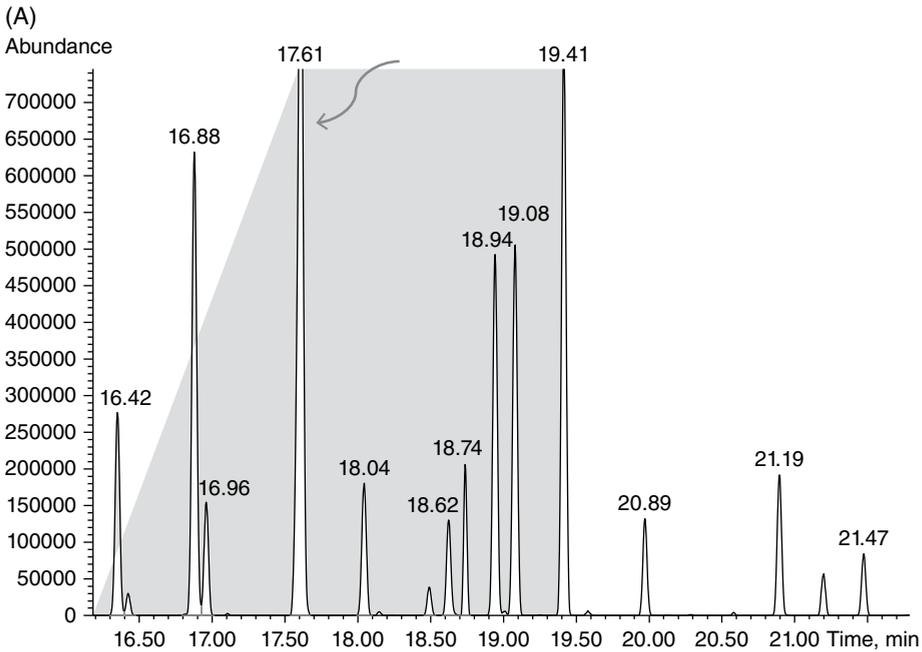


Figure 3.21 Mass spectra (electron ionisation, 70 eV) and typical fragment-ions of isomeric esters: **A.** Methyl ester of 4-amino benzoic acid, and **B.** Methyl ester of 2-amino benzoic acid (methyl anthranilate). Fragment-ion at m/z 119 in methyl anthranilate mass spectrum is a product of *ortho*-effect rearrangement, that is, methanol CH_3OH elimination.



(B)

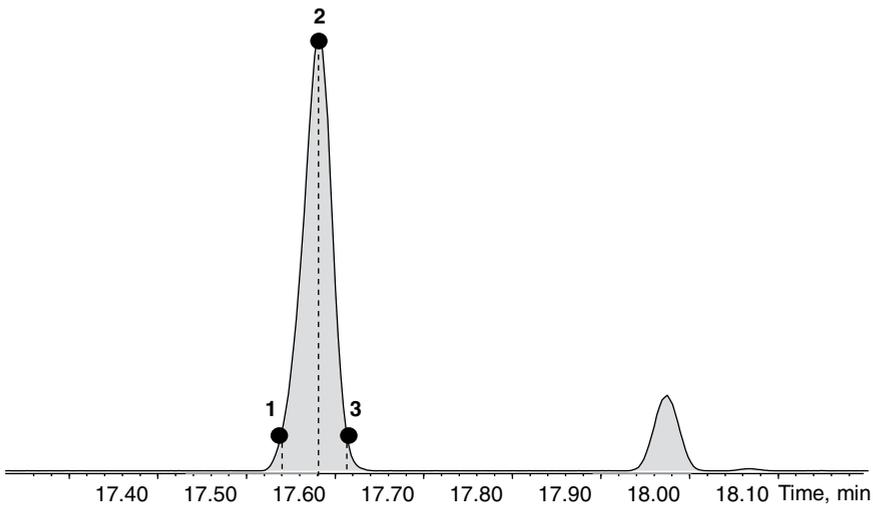


Figure 3.22 **A.** Part of the chromatogram (GC/MS, TIC, electron impact, 70 eV) of the volatile fraction isolated by *in vivo* HS-SPME from *Moringa oleifera* flowers (Moringaceae family). Polar column (PEG, 60 m). **B.** GC peak at $t_R = 17.61$ min, is symmetric and apparently homogeneous, possibly representing just one compound. Different points of the peak in which the mass spectra were obtained. **C.** Mass spectra of three different substances, that is, butyl isothiocyanate (1), hexyl acetate (2) and *p*-cymene (3), obtained at the beginning, in the middle and at the end of the peak. **D.** Extracted ion chromatograms (EIC) of the base-peak ions in the mass spectra of these three substances and their GC areas, which permit to find approximately the ratio of these substances present in the co-eluted mixture.

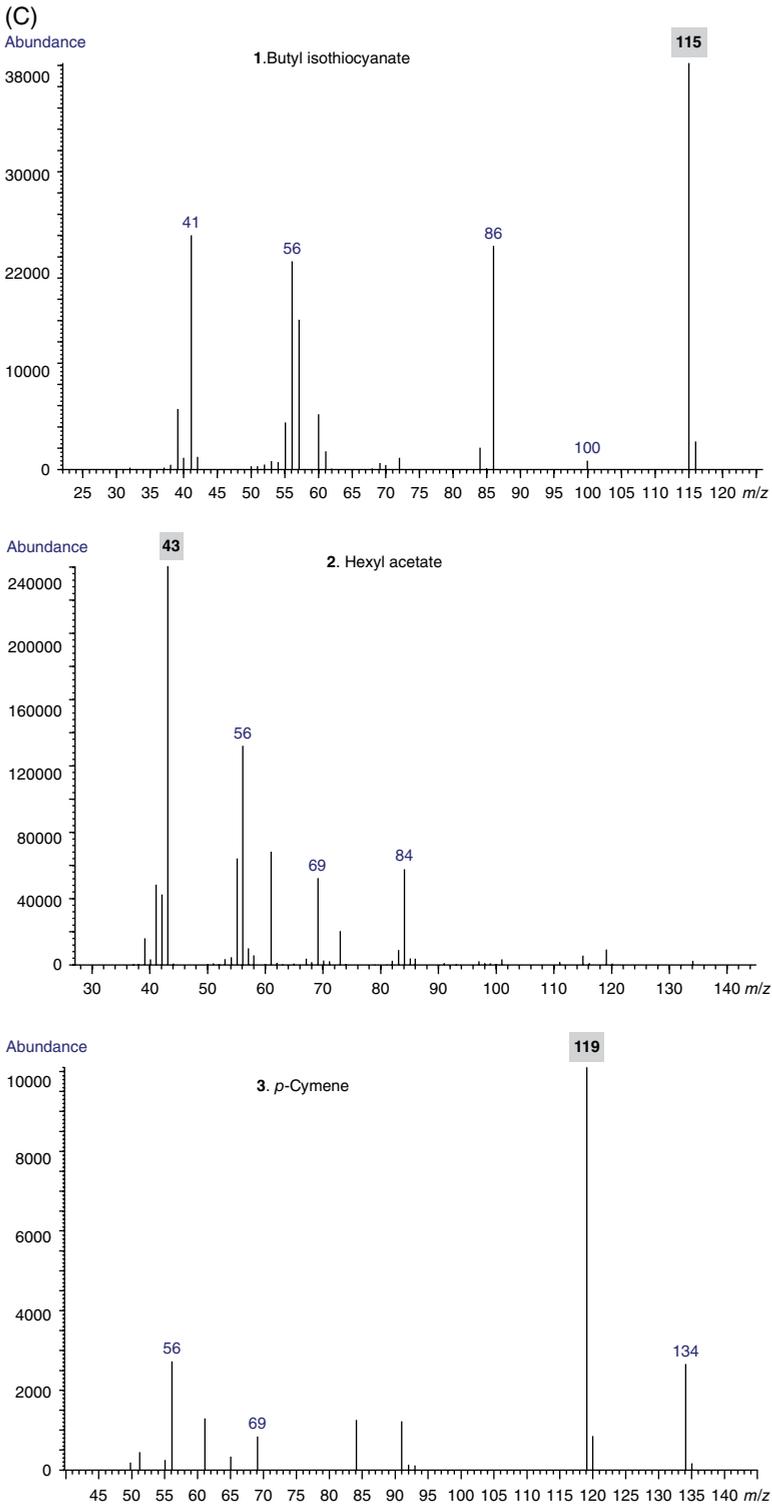


Figure 3.22 (Continued)

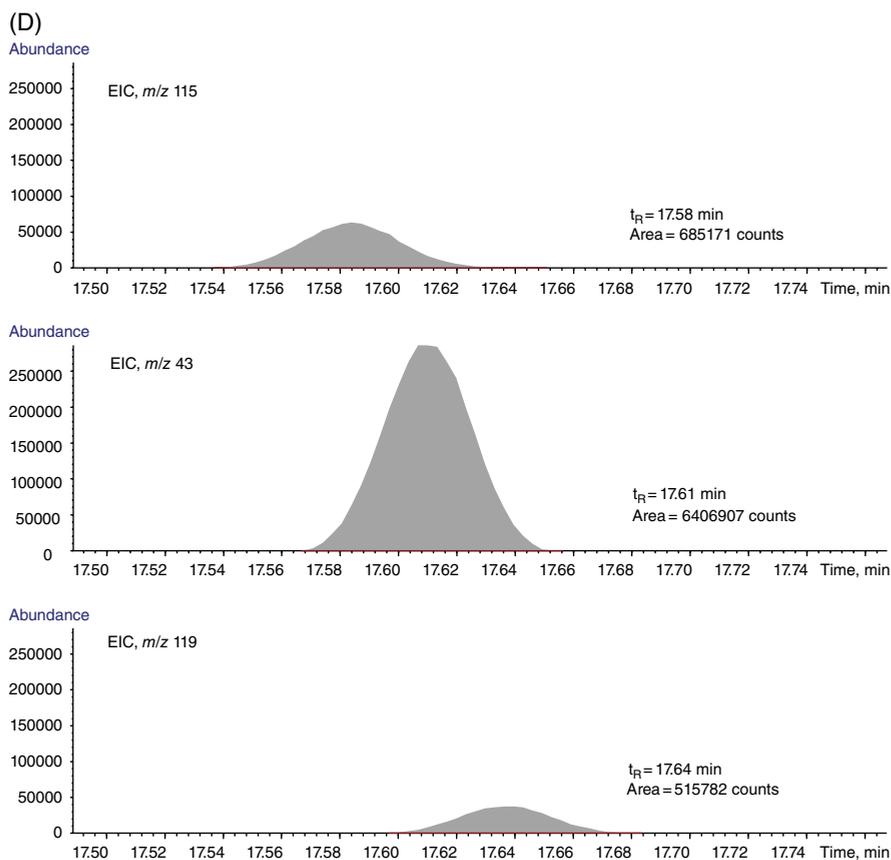


Figure 3.22 (Continued)

it depends on the amount and type of analyte-molecules entering, but also on background molecules present (air, moisture, solvents, stationary phase bleeding products, chemical noise, pollution, plasticisers, *etc.*).

The homogeneity of each chromatographic peak can be monitored by proving the equality or not of mass spectra taken at different points in the elution window of one or more substances (Figure 3.22).

For chemical structure elucidation, each mass spectrum is studied by analysing its general appearance, presence of molecular ion, isotopic pattern, the occurrence of nitrogen in the molecule (the 'nitrogen rule'), the characteristic ions, and the fragmentation pattern. In most cases, LRI measured in polar and non-polar stationary phases are compared with published data; mass spectra are compared with those of mass spectra libraries. The result is a tentative identification. The confirmatory identification (absolute) of a compound by GC-MS is only possible when the same operational conditions of the equipment are used to analyse the certified standard (reference substance).

When only some characteristic or 'diagnostic' ions (generally, 1–3) are recorded in the mass spectrum of a substance, the processed partial ion current *versus* time is called mass fragmentogram; the chromatographic profile contains exclusively peaks of the

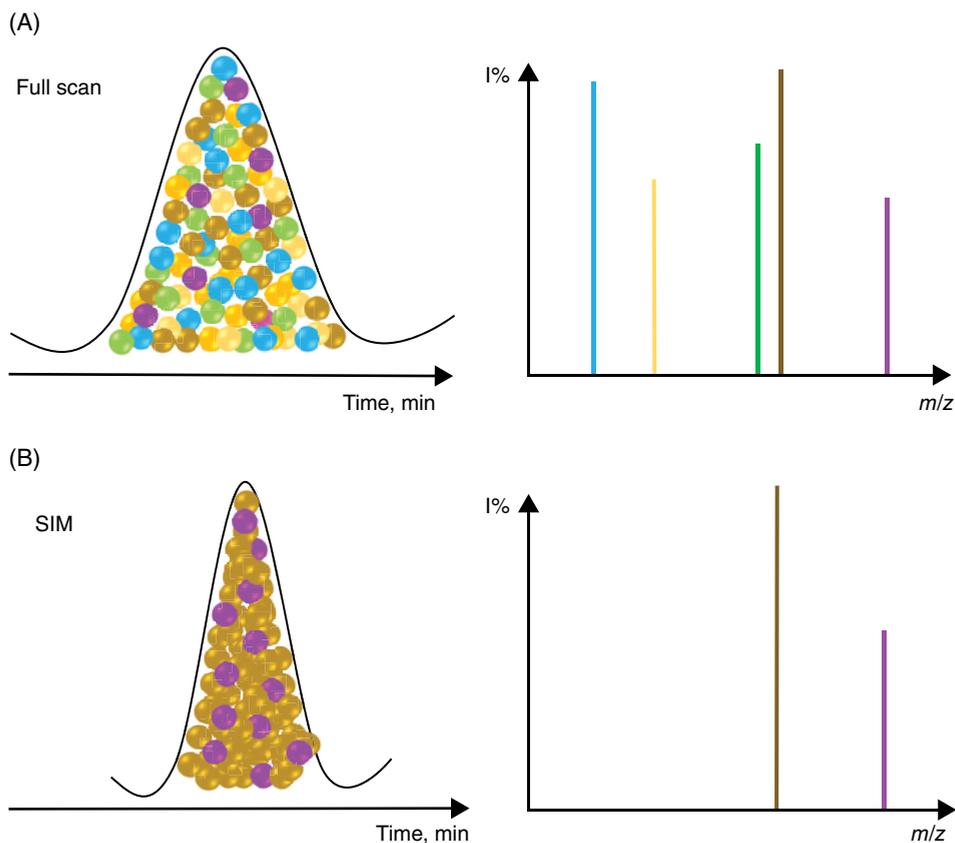


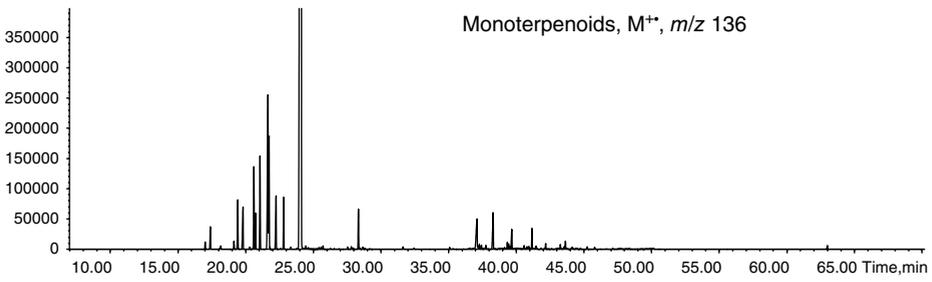
Figure 3.23 Comparison of two GC-MS acquisition modes. **A.** Full scan mode is when all ions are registered and their full mass spectra, which permit compound identification, are obtained. Reconstructed or total ion current (TIC) is measured and plotted as a function of time and constitutes a chromatogram. **B.** Selected ion(s) monitoring (SIM) is carried out when only one or few 'diagnostic' ions are registered and permits to selectively detect a compound or several compounds which share the same ions in their mass spectra. Mass fragmentogram is obtained in SIM-mode. **C.** *Cannabis sativa* inflorescence hydro distilled essential oil, analysed by GC-MS operated in SIM mode, using m/z 136 and m/z 204 ions for selective detection of monoterpene and sesquiterpene molecular ions, M^+ , respectively. **D.** *Brugmansia suaveolens* (Solanaceae family) CO_2 -supercritical fluid extract (SFE), analysed by GC-MS in full scan-mode and SIM-mode. 'Diagnostic' ions at m/z 94, 138 and 303 were used for selective detection of scopolamine.

substances of interest, in whose mass spectra appear the preselected ions. This method of acquiring partial ion currents is known as selected ion monitoring or SIM. When SIM acquisition mode is used, the mass detector becomes a selective chromatographic detector (Figure 3.23). Further increments in detection specificity usually require the use of more than one analyser, that is, a multidimensional configuration (in time or in space), known as tandem configuration.

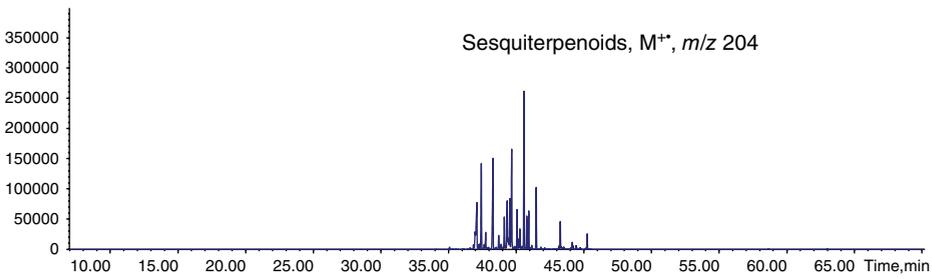
Mass selective detectors may be classified into two groups. First, what are called scanning analysers. These include sector analysers, for example, magnetic deflection of one or two sectors (magnetic and electrostatic fields), or quadrupole analyser, which is one

(C)

Abundance

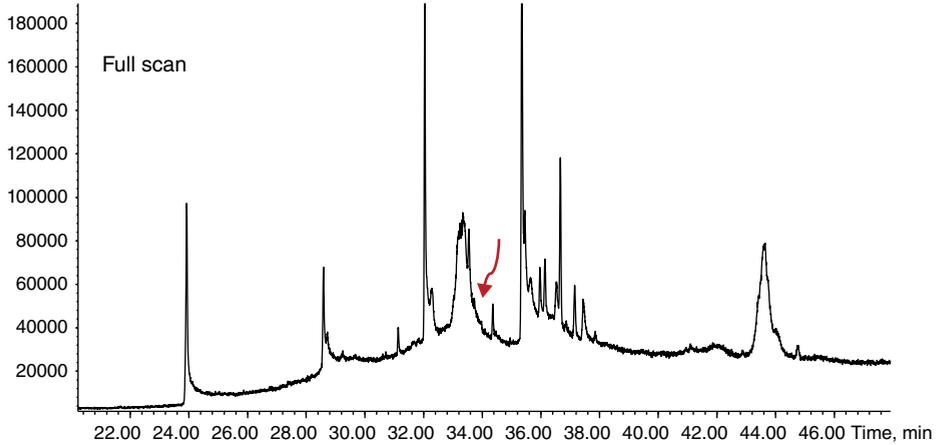


Abundance



(D)

Abundance



Abundance

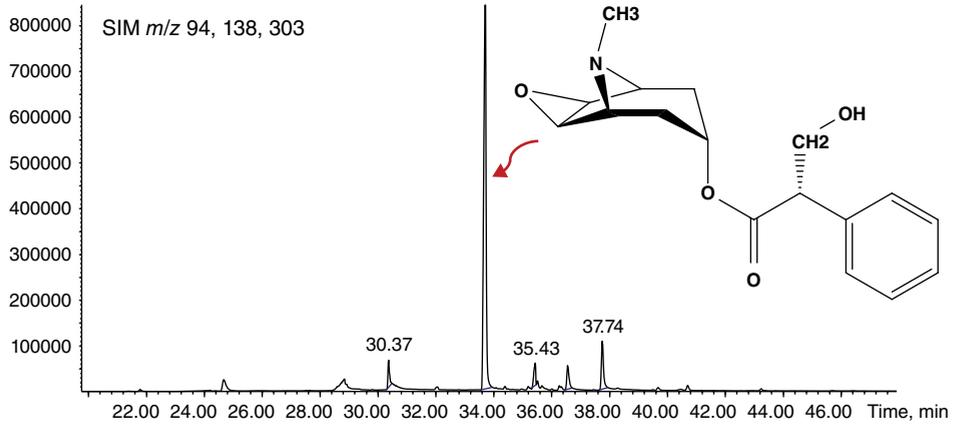


Figure 3.23 (Continued)

of the most frequently used in GC-MS. The magnetic sector analyser was historically the first mass analyser used, but is not employed in current GC-MS instruments. The second group consists of analysers with simultaneous transmission of ions. They include the time-of-flight (TOF), different types of ion traps (IT), Fourier transform mass analysers (FT-MS), namely, mass spectrometers of ion-cyclotron resonance (ICR-MS), and the orbitrap, which also uses a Fourier transform to obtain the mass spectrum from the frequencies of the ions moving in an electrostatic potential; the latter two have lately gained popularity in the field of coupled techniques with both gas and liquid chromatography, although the orbitrap has the advantage of providing high resolution at lower investment and operation costs.

When one-dimensional GC-MS analysis is used to quantify and identify EO components, the following conditions should be satisfied: (1) Use of longer (50, 60 m) capillary columns; (2) Analyses on two capillary columns with different stationary phases (e.g., DB-1 or DB-5, and DB-WAX); (3) Obtainment of EI (70 eV) mass spectra and their comparison with those of several mass spectral databases (e.g., NIST, Wiley, Adams); (4) Calculation of the linear retention indices for the polar and nonpolar stationary phases and (5) Confirmation of structural assignments with certified standard compounds. This set of parameters permits the confirmatory identification of the mixture components (Molyneux and Schieberle, 2007). Ensuring peak homogeneity is very important in GC-MS analysis because incorrect structural assignments may result if various substances coelute.

An important application of EO GC-MS analysis is quality control of commercial products that use them as ingredients. Individual component identification is relevant, but does not play a central role in these applications. Chemometric techniques, such as principal component analysis (PCA) and partial least-squares, are powerful tools for comparing the complete GC-MS profile of the volatile fraction of various samples to establish their similarity. Hu *et al.* (2014) used this approach to distinguish *Curcuma longa* EOs of four different geographic origins. Turmerone, *ar*-turmerone and zingiberene were selected as discriminating constituents from the GC-MS fingerprint data of 24 different turmeric oils. Three different chemotypes of *Lippia origanoides* were clearly distinguished in the PCA results of the EO composition tables built from their GC-MS profiles (Stashenko *et al.*, 2010).

3.4.1 Tandem Methods

Like a one-dimensional chromatographic system (single column) that can reach capacity limits to separate components of a complex mixture, 'one-dimensional' mass spectrometry (with a single mass analyser) may also reach their limits of mass range, sensitivity, resolution and, mainly, amount of information required for molecular structure elucidation. When very few signals appear in the mass spectrum, predominantly represented by protonated (or deprotonated) molecular ions (soft ionisation techniques) and some cluster ions and not fragment ions, it is very difficult to reliably establish the molecular chemical structure. The mass spectra of extracts from biological samples, food, soil, and so on, may contain excessive chemical noise. This prevents achieving the specificity required to detect and identify the analytes of interest in a reliable manner, especially, for complex mixtures with a high number of interferences or impurities.

Product ions, precursor ions, the reactions that link two related ions, are the features that can be studied with the tandem configuration. The loss of a neutral fragment can be monitored as well, amongst other options. Tandem MS/MS analysers includes the triple quadrupole, designated by its acronym QQQ or QqQ. Hybrid MS/MS configurations involve the combination of several analysers with different operating principles. A quadrupole (Q) or an ion trap (IT) may be interfaced with a magnetic sector analyser (B) alone or in conjunction with a time-of-flight analyser (TOF), or an electrostatic analyser (E). This originates different hybrid tandem systems. Q-TOF, IT-TOF, EBE, EBEB, B-QI-Q2, QEB, TOF-B, EB-TOF, EBE-TOF and QBE are examples of possible analyser combinations. Of course, the cost and complexity of the instrument and its operation increase considerably. However, the quantity and quality of the analytical information obtained, the degree of reliability and specificity, also experience substantial growth.

The MS/MS technique is a very valuable analytical tool when high selectivity or specificity are demanded. One example of these situations is the appearance of high chemical noise in GC-MS spectra. A second type of cases is the co-elution of characteristic ions with isobaric impurities. A related situation happens when additional confirmatory evidence is needed in the interpretation of a mass fragmentogram obtained in SIM mode. Unambiguous structure elucidation sometimes requires the generation of additional fragments or the study of parent-daughter ions, which is possible with tandem arrangements. The determination of multiple pesticide residues, the detection of petroleum biomarkers, the quantification of anabolic steroids or other doping agents, typically demand high sensitivity and high specificity, which can be achieved with tandem configurations. The various experiments now possible with a triple quadrupole provide specific information based on the determination of product and precursor ions, or the exclusive monitoring of selected reactions between ions.

In chromatographic systems (GC or LC) coupled to a mass selective detector, the latter is a reliable tool to elucidate the chemical nature of the substance thanks to the fine spectral details it affords. The MS can work, depending on the mode of acquisition of ionic current, as a universal detector (full scan mode), a selective detector (SIM mode) and as a system of highly selective and sensitive (specific) detection. The mass detector becomes a specific detection system when it is in a multi-dimensional configuration, tandem MS/MS. Amongst the tandem mass spectrometric detection systems, a polyfunctional, relatively easy to operate and comparatively low cost (market price, maintenance, ease of handling, *etc.*) configuration is the triple quadrupole. This is a mass spectrometer or detector which has three quadrupole analysers, and operates as detection system for gas chromatography or liquid chromatography, in universal, selective or specific regimes, according to the acquisition mode of the ion current produced during the ionisation and fragmentation of target analyte molecules.

One of the most interesting ionic current acquisition methods made possible by the triple quadrupole is multiple reaction monitoring, MRM. The proper use of its advantages permits to obtain high selectivity and sensitivity in LC or GC. In the SIM experiment it is possible that a signal from the background chemical noise coincides with that of the selected ion. This not only reduces the sensitivity, but the reliability of the detection, since both false-positive or false-negative detections become possible. In order to avoid these problems, the MRM experiment monitors transitions between pairs of ions (precursor and product), rather than characteristic ions. For a given precursor-product

transition, the m/z values of precursor (f_1) and product (f_2) ions are known, from the previous acquisition of product ion spectra and precursor ion spectra. The first quadrupole (Q_1) operates in SIM mode to act as a filter to allow passage only to precursor ions f_1 . The second quadrupole (q_2) acts as a collision chamber in which fragmentation takes place. The third quadrupole (Q_3) operates in SIM mode to permit passage of only product ions f_2 . Thus, a signal from the electron multiplier at the end of Q_3 indicates that indeed both f_1 and f_2 exist and have this precursor-product relationship. Both f_1 and f_2 should be stable, abundant ions in the mass spectrum of the analyte. Monitoring the 'precursor-product' transition (reaction) almost eliminates the probability of random coincidences between the analyte signals and those of the chemical background and increases the signal/noise ratio (Figure 3.24). The registration of two independent transitions and the coincidence of chromatographic retention times, constitute the unequivocal confirmation of the occurrence of a target analyte in a complex mixture.

The fast GC-QqQ analysis of mandarin, lemon, sweet orange and bergamot EOs provided spectroscopic data to identify oil components and to quantify three specific preservatives (*o*-phenyl phenol, butylated hydroxytoluene and butylated hydroxy anisole) (Tranchida *et al.*, 2013). Data acquisition lasted only 17 min per sample; full scan spectra were used for qualitative purposes and MRM data for the quantification of preservatives. The specificity of the QqQ system may be used for the determination of multiple pesticide residues or particular, distinctive constituents. For example, Fillatre *et al.*

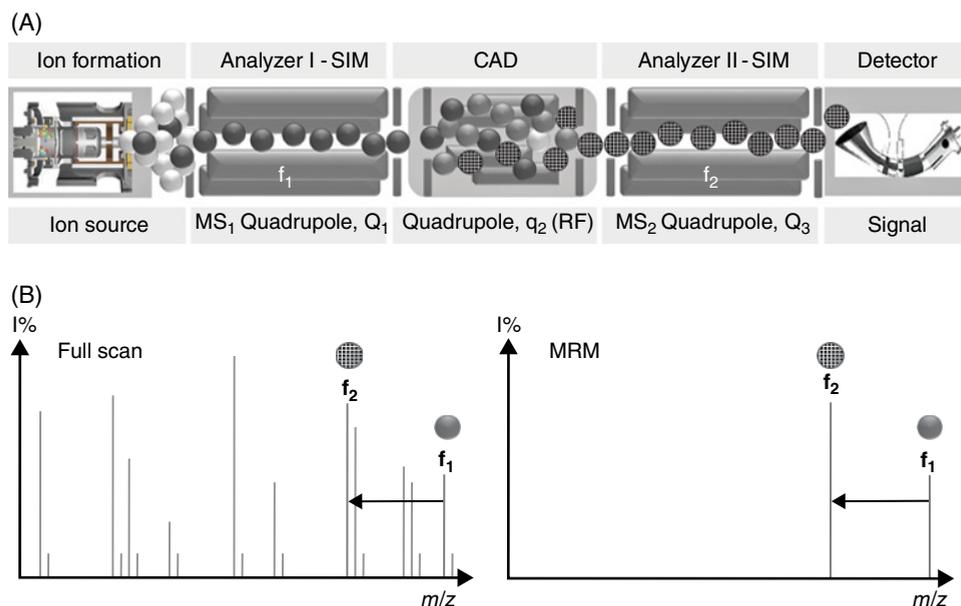


Figure 3.24 Tandem (in space) mass spectrometry: triple quadrupole, QqQ. **A.** Multiple reaction monitoring, MRM. Precursor ions (f_1) are selected in the analyser I (Q_1), operated in SIM-mode and are directed to the collisionally-activated dissociation cell (q_2 , operated only with radiofrequency, RF), where they are fragmented; the Analyser II, Q_3 , filters the selected product ions (f_2); the signal of the ion transition (reaction) $f_1 \rightarrow f_2$ is monitored as a function of time. **B.** Comparison of two mass spectra obtained in full scan acquisition mode and MRM-mode; the f_1 and f_2 ions are highlighted in the first (left) mass spectrum.

(2014) reached quantitation limits of $1 \mu\text{g}/\text{kg}$ in the determination of residues of 256 different pesticides in lavender oil. The EO was gently heated while it was evaporated with a nitrogen stream and the residue obtained was analysed by LC-MS/MS in MRM mode. Vallverdú-Queralt *et al.* (2014) used MRM to quantify 11 polyphenols in spice extracts obtained with solid-phase extraction and later subjected to LC-MS/MS analysis. Principal component analysis of this compositional information permitted to classify correctly the spices as cumin, cinnamon, thyme, oregano, rosemary or bay, based on the proximity of the sample coordinates to the areas corresponding to these spices when represented in the plane formed by the first two principal components.

3.4.2 Multidimensional and Comprehensive Techniques

Essential oils constitute a formidable challenge for gas chromatographic analysis, due to the diversity of chemical structures and wide range of relative amounts in which the constituents are present. When columns with different stationary phase polarity are used some substances may coelute in only one of the columns (non-polar or polar). An additional complication is that some EO constituents have very similar mass spectra and this prevents the use of mass-fragmentograms as separation tools.

The concept of doing the chromatographic analysis on two columns of different polarity has led to multidimensional chromatography. In the 'heart-cutting' arrangement, two columns are connected via pneumatic or microfluidics commuting devices, which for fixed time periods direct the eluting flow from one to the other column. This permits to use a second stationary phase to separate the peaks of completely or partially coeluting substances (Figure 3.25). At least two detectors are required and there may be three columns in the same or separate chromatographic ovens (Marriott *et al.* 2012). A very important configuration uses a chiral stationary phase in the second column. The

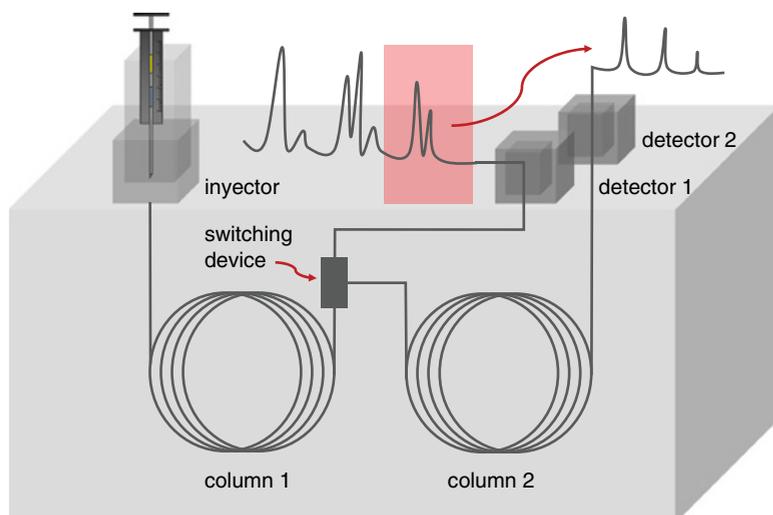


Figure 3.25 General scheme of the multidimensional gas chromatography (MDGC) system with two orthogonal columns (polar and non-polar), connected through the switching device, which performs the heart-cutting, diverting the eluting compounds from the first to the second column.

resulting multidimensional GC (MDGC) technique has been effective in the chiro-specific analysis of natural flavours and EOs, particularly, in authenticity determination. The detection of frauds or adulterations is a continuous preoccupation in the food field. Since many biosynthetic processes are stereospecific, the determination of enantiomers or enantiomeric compositions permits to characterise a product of natural origin (Cagliero *et al.*, 2016). At the same time, it allows the detection of synthetic racemic products used as replacements for EOs and fruit flavours. Mosandl *et al.* (1991) illustrated the use of a chiral stationary phase containing heptakis (2,3,6-tri-*O*-methyl)- β -cyclodextrin as chiral selector, to differentiate α -pinene, β -pinene and limonene in complex matrices, thanks to chiral-MDGC analysis. Since this enantioselective analysis has become an important tool in quality control laboratories, many efforts have been made to reduce analysis time while maintaining high reliability.

One of the approaches to reduce experimental time consists of using chromatographic columns of smaller diameter and shorter length. This can be combined with innovations in the interface that transfers the flow from the first to the second column. Mondello *et al.* presented in 2006 a first application of a new fast MDGC-FID technique that produced the same results of a conventional MDGC system, but in one fifth of the time. Sciarrone *et al.* (2010) used the analysis of volatile chiral compounds in mandarin EO to compare the results obtained with one-dimensional chiral GC-MS and with chiral-MDGC. They concluded that in several cases 1D-GC produced errors (mainly due to coelution) and MDGC remains the primary option for these analyses due to its enhanced separation power. Commercial MDGC instruments are available from several brands, with simple operational protocols and well-tailored software. Although in many situations this technique is the best choice in many respects, users have preferred GC-MS and comprehensive chromatography (*vide infra*). In the 2000–2010 period, the amount of scientific articles on MDGC that were published in the *Journal of Chromatography A*, was seven times smaller than the number of articles dealing with comprehensive chromatography (Tranchida *et al.*, 2012).

In total or comprehensive chromatography, abbreviated GCxGC, every single segment of the eluting flow is transferred to the second column during the whole chromatographic run. The multiplication sign evokes the fact that the peak capacity of this technique is the product of the peak capacities of the two columns employed ($N \times N$). An example of the results of this increased resolution is the analysis of ylang-ylang EO. While the 1D-GC analysis of this oil revealed the presence of 51 constituents (Stashenko *et al.*, 1996), the GCxGC technique permitted the identification of 161 different compounds (Brokl *et al.*, 2013). During GCxGC operation, a modulator (there are several versions available) collects a short segment of the eluting flow from the first column and transfers it to the second column. The transferred portion should transit the complete length of this second column before the next flow segment is admitted. This explains why the second column is not much longer than 1 m (Marriott *et al.*, 2001; Tranchida *et al.*, 2016).

In contrast with multidimensional chromatography, only one detector is needed in GCxGC. However, this detector should be fast enough to provide sufficient data points for the correct digitalisation of the chromatographic peaks. The chromatographic columns may reside in one or separate ovens. The modulators differ in the approach employed to perform the fractionation of the flow from the first column (1D, 25–30 m) into consecutive segments which are transferred to the second column (2D, microbore, 1 m)

with or without cryo-concentration. The elution time in the second column (short, small diameter) lasts several seconds and should be complete when the next flow segment is transferred. Since the second column is connected to the detector, the latter should have a very high speed of data collection and processing (MSD, FID, μ -ECD).

The first decade since the appearance of functional modulators, was dominated by GCxGC-FID instruments. However, the need for reliable identification demanded a third coordinate, which was provided by MS detection. Although the TOF mass analyser has a relatively high cost, it is the best choice for fast response (Mondello *et al.*, 2008). Figure 3.26 presents the case of limonene and β -phellandrene separation to illustrate the resolution achieved in the GCxGC analysis of *Cannabis sativa* EO.

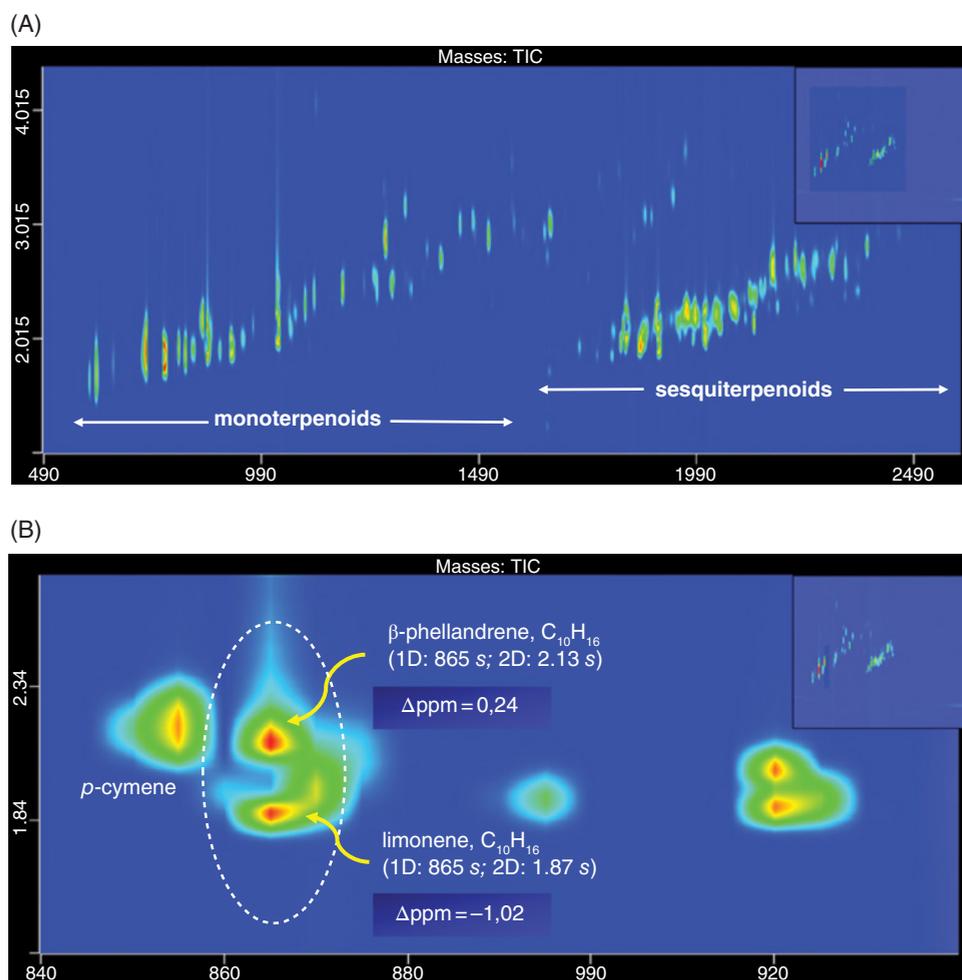


Figure 3.26 **A.** GCxGC chromatogram (TIC), obtained with high-resolution time-of-flight analyser (HRTOF-MS), of *Cannabis sativa* inflorescence essential oil. Cryogenic dual jet/loop modulator; secondary oven installed in the main GC oven; 1D – first column: Rxi-5MS, 30 m, L, 250 μ m, ID, 0,25 μ m, d_f and 2D – second column: Rxi-17Sil MS, 2 m, L, 250 μ m, ID, 0,25 μ m, d_f ; modulation time: 5 s. **B.** Fragment of the TIC showing the separation of limonene and β -phellandrene in the second dimension (2D, polar column).

Since peaks are digitised for the data handling systems, at least 10–12 data points should be collected across a peak width at half height in order to have an accurate representation (Poole, 2003). The second dimension of GCxGC affords peaks with widths of 0.01 to 0.1 s. Thus, the detection system should have a data acquisition rate of 100 Hz to achieve the 10 points required for a 0.1 s-wide peak. Blase *et al.* (2014) compared the results of using 10, 50, 100 and 200 Hz detector acquisition rates in GCxGC of a multi-component mixture. The change from 10 to 200 Hz caused a nearly twenty-fold increase in peak area, an eight-fold increase in peak height, and a five-fold decrease in peak width. The latter reduction immediately results in much higher chromatographic resolution. The detection limit determined for 200 Hz operation was 19 times lower than that calculated for 10 Hz sampling rate. These results explain why TOF is the preferred analyser in GCxGC-MS. Most of these instruments use low-resolution TOF analysers, which provide unit-mass resolution spectra, appropriate for searches in commercial databases. The analysis of hop EO is a good example of the type of solution that GCxGC brings about in the characterisation of complex volatile mixtures (Roberts *et al.* 2004). Since hop is a major beer flavour determinant, brewers require a reliable distinction of hop varieties. However, 1D-GC analyses give contradictory results because many of the oil constituents co-elute. The high separation provided by GCxGC-TOF permitted to identify 119 different components in this EO, 45 of which had not been reported previously. A spectral acquisition rate of 60 Hz was used.

However, the quadrupole is a more widely available and simpler device. Thus, there is a growing number of reports on GCxGC analyses that use a quadrupole as mass analyser. Shellie and Marriott (2003) analysed *Pelargonium graveolens* EO using a 20-Hz spectral acquisition rate that was achieved by reducing the quadrupole mass scan range to 188 u. They reported a satisfactory EO characterisation and showed the feasibility of calculating key parameters for geranium oil quality such as the citronellol/geraniol proportion and the amount of 10-epi- γ -eudesmol. Nevertheless, the scarcity of points per peak constitutes a limitation for quantitative analysis. A more recent fast-scanning quadrupole detector, capable of reaching a scan speed of 20,000 amu/s, was employed in the determination of allergens in perfumes. After testing spectral scan speeds of 25 and 33 Hz, Purcaro *et al.* (2010) validated a GCxGC method for allergen quantitation in which the quadrupole detector provides a 50-Hz sampling frequency in the 40–330 m/z mass range. The number of data points (spectra) acquired per peak for representative analytes, such as linalool, eugenol, linal and benzyl salicylate, were 18, 24, 21 and 24, respectively. These results showed that a fast-scanning quadrupole is a viable option of quantitative GCxGC analysis.

Although mass spectrometric detection is a very useful resource for compound identification, even in the case of high-resolution MS detection it is possible to have situations in which isomeric compounds cannot be differentiated because they have very similar fragmentation patterns. Thus, even if the mass spectrum is available, the uncertainty remains. In these situations it is necessary to use additional identification criteria, which in 1D-GC are represented by retention indices. The analogy in GCxGC is constituted by the pair of retention indices, 1I and 2I , each calculated for the stationary phase used in the corresponding dimension. The determination of retention indices in the first dimension follows basically the same procedure and formulas of 1D-GC (Section 3.2). The projections on the 1D time axis of the sample and an alkane homologous series run under the same chromatographic conditions are used to obtain the retention times employed in

eqns. 3.1 or 3.2. However, a similar approach cannot be applied to the second dimension because the retention times depend on temperature and the temperatures of the elution point are different for target analytes and reference alkanes. Von Mühlen and Marriott (2011) reviewed this subject and presented several manners in which the observed retention times and elution temperatures may be related to a retention index.

An alternative methodology of relatively simple implementation was recently demonstrated by Jiang *et al.* (2015) in the GCxGC-high resolution quadrupole-TOF-MS analysis of saffron EO. The central step in this method is the use of stepwise isothermal oven temperature programming for GCxGC runs of multiple injections of the reference alkane series. A series of volatility curves is obtained, which permits the calculation of the retention indices in the second dimension for the target analytes. In the saffron volatiles analysis presented, several isomeric compounds had similar fragmentation patterns which led to ambiguous results in spectral database searches. These cases were positively resolved, thanks to the use of the set retention indices in both dimensions. The combined use of spectral data and retention indices in both dimensions strongly reduces the uncertainty in tentative compound identification.

3.5 Isolation of Individual Components or Enriched Fractions

The identification of EO components which are present in low concentration is doubtful by the fact that even though their chromatographic peaks may be well-resolved, their mass spectra are not of sufficient quality for a reliable identification. This could be caused by the interference from the signals of the background spectrum or by insufficient instrumental sensitivity. A solution that has been used in these cases is to employ heart-cut-MDGC to isolate fractions of the EO containing these minor components. A cold trap is installed between the two chromatographic columns, the sample is injected several times, and several heart-cuts are accumulated in the trap. The resulting fraction is subsequently analysed as a new sample and higher S/N is obtained in its mass spectra (Bicchi *et al.*, 1989). Several variations of this approach have been employed, using heart-cut MDGC with one or two columns and a cryotrap, plus a commutation valve to direct the flow to the trap, the column, or the detector. If obtaining a higher-quality mass spectrum of the target analyte is not sufficient to reach the proposed goals, the additional information is sought with NMR spectroscopy, or vapor-phase FT-IR spectroscopy. However, these techniques impose an increase to μg and mg scale in the required amount of purified compound.

In other research situations, the organic synthesis of a particular substance may be complex or inefficient and the isolation from its natural source is a better alternative in time and costs. An additional consideration is the environmental impact of using considerable amounts of organic solvents, something that discourages the selection of techniques based on separation in the liquid phase. Considerable advances have been made in preparative gas chromatographic techniques to isolate almost pure individual components in up to mg amounts (Sciarrone *et al.*, 2015).

A capillary NMR tube was used by Nojima *et al.* (2011) to collect 50–250 ng amounts of purified volatile compounds, eluted with deuterated solvent from a short (20 cm), coating-free capillary (0.53 mm ID) in which the purified compound

had been transferred in successive chromatographic runs. This 1D-preparative GC method usable with either a polar or a non-polar mega-bore column (30 m, 0.53 mm ID, 1- μ m film thickness) was applied to the isolation of geranyl acetate, from which ^1H NMR and COSY spectra were obtained. However, coelution is a main obstacle to the use of preparative 1D-GC to satisfactorily isolate mixture components. This is the main reason for the more common application of multidimensional techniques.

Eyres *et al.* (2008) successfully isolated geraniol from a mixture of peppermint, spearmint and lavender EOs, in whose chromatogram it coeluted with linalyl acetate, carvone and pulegone. The MDGC system employed included two capillary columns (15 m, 0.32 mm ID, 1 μ m d_f) of different polarity connected with a longitudinally modulated cryogenic system (LMCS) and a short column loop which acted as temporary storage for the heart cut. The material collected in the loop was directed to the second column. At the end of this column a microfluidics Deans switch transferred to a cold trap a segment of this flow, that corresponded to the separated compound. The amount of geraniol collected after 10 injections was sufficient to obtain ^1H NMR, and gradient COSY spectra, while that obtained after 100 injections permitted two-dimensional NMR experiments such as gradient heteronuclear single quantum correlation spectroscopy (gHSQC), and gradient heteronuclear multiple bond correlation spectroscopy (gHMBC). The same research group subsequently developed a simpler preparative MDGC arrangement without the loop and the LMCS, which was tested in the isolation of menthol and menthone from peppermint oil (Park *et al.*, 2012).

Several variations of the MDGC technique for pure compound isolation have been published. An interesting development has been the use of LC as initial fractionation step which allows the injection of a larger amount of target analyte into the preparative GC setup. Pantò *et al.* (2015) showed that with this three-dimensional preparative system, individual components with abundance below 5% in their original natural source could be isolated in mg amounts with purity higher than 87%. The use of a packed LC column as first separation step permitted the injection of relatively large sample amounts (10- μ L) into the first preparative GC, which used a macro-bore column. The overloading of this column was endured by the system, which thus obtained a high productivity manifested in the isolation of mg amounts of target analyte accumulated from several chromatographic runs that lasted about 1 h each. The three-dimensional preparative system consisted of three gas chromatographs connected by means of three Deans switching devices, the last one being used for delivery of the isolated analyte to the cryotrap.

3.6 Conclusions

Essential oils are used in many fields all over the world and their continuously increasing use generates growing demands for their comprehensive chemical analysis. The analytical challenges are diverse and the development of new and more powerful methodologies for EO analysis is a very active research field. Comprehensive gas chromatography, chiral analysis, elemental composition determination and trace component detection have experienced rapid development in recent years. Their analysis may be aimed at authenticity assessment, or the determination of chiral components, or the identification of components with certain specific biological activity or the

determination of the main aroma-impact compounds. Basic research on plant biodiversity and the continuous search for new EO applications in consumer products, require their complete and detailed chemical characterisation. Chromatography lies at the core of the large majority of successful instrumental techniques and methods developed to address all these different needs. The separation of these complex mixtures over one or more stationary phases precedes the transfer to the detection system, which in many cases is spectroscopic/spectrometric in nature. The spectral information constitutes an additional dimension in the space within which the EO components are being characterised. Mass spectrometry is the most commonly used detection system, particularly when the main interest is substance identification. Routine quality control is regularly performed with 1D GC or GC-MS. However, MDGC and GCxGC are increasingly used for routine analysis, not just for basic research. It is worth to highlight that preparative gas chromatographic methods developed recently, particularly those based on MDGC, permit to make available almost any EO constituent in almost pure form, in up to mg amounts, and to identify it by NMR or other spectroscopic methods.

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4

Chemical Composition of Essential Oils

Hassan Eslahi, Nafiseh Fahimi and Ali Reza Sardarian

Department of Chemistry, College of Sciences, Shiraz University, Iran

4.1 Introduction

Essential oils (Burt) (ethereal or volatile oils) are natural volatile compounds that large amount of them are produced from plant raw materials or plants organs like flowers, seeds, bud, leaves, fruits, wood, roots, barks and twigs. They give a distinctive flavour, odour or scent to an aromatic plant. For example, citrus-derived essential oils included wide group of fruits from genus citrus, are the most well-known natural EOs and account for the largest spectrum of commercial available natural fragrances and flavours (Sawamura, 2010; Baser & Demirci, 2007; BasDudarevaer & Buchbauer, 2010). Essential oils are safe for human and animal usages and have been considered as ethereal or volatile oils, occur in eatable, herbal and medicinal plants (Benchaar *et al.*, 2008; Do *et al.*, 2015).

EOs are obtained from more than 17,500 aromatic flora or plants bearing many Angiospermic families such as Rutaceae, Lamiaceae, Myrtaceae, Asteraceae and Zingiberaceae (Regnault-Roger *et al.*, 2012). They are by-products observed from plant metabolism are referred to as secondary metabolites of volatile plant.

EOs can be applied in various cases including pharmaceutical and health industries (as antiseptic, antibiotic, antiparasitic and anti-inflammatory agents), chemical industries, food sectors (as preserving and flavourings agents), the cosmetics and perfume industries (as fragrances, antibacterials and aromatherapeutic agents), and also, agriculture pathogenic agents such as phytopesticides, agricultural pests and weeds or bioinsecticide and biofungicide. Their applications have been increasing due to wide requirements for pure natural ingredients, which caused these materials be inseparable part of our life. An increasing demand for them have been observed in the field of adulteration (Baser & Demirci, 2007; Vergis *et al.*, 2015; Edris, 2007; Charai *et al.*, 1996).

There are many reports in the literature have contained useful information about the composition of different EOs exhibit the majority of economically valuable EOs components (Bauer *et al.*, 2001; Diao *et al.*, 2013; Lee *et al.*, 2013; Bailen *et al.*, 2013; Ali *et al.*, 2015; Viuda-Martos *et al.*, 2010; Avonto *et al.*, 2016). Essential oils are complex materials and multi-component systems consisting of many chemical components (Li *et al.*, 2015),

which may be so knotty to identify all types of them. For example, there has been found total number of 36 components of EOs from *Murraya* genus (You *et al.*, 2015). Moreover, exact analysis by analytical instruments are needed to investigate and find the chemical compositions of EOs (Akkari *et al.*, 2015; Morshedloo *et al.*, 2015; Memarzadeh *et al.*, 2015). Herein, there are inconsiderable researches in the recognising of some cases such as *A. hedinii*, which was investigated by Zhigzhitzhapova and co-workers (2014) for the first time.

The wide range of EOs is somehow complex based on their variability, activity and nature in structure make it difficult to find consistent positive responses in the enhancement of production efficiency in ruminants (Benchaar *et al.*, 2008). The major structures of interesting EOs are summarised by (Bauer *et al.*, 2001). Because of different methods and the various factors effect on, EOs are complex matrices containing a lot of compounds with several functional groups and structures (Table 4.1) (Benchaar *et al.*, 2008).

EOs are classified into non-volatile, semi-volatile, and volatile compounds according to their nature. EOs are obtained from plants and will consumed as highly volatile alarm-type structures, which must spread into the surrounding air, through to more compounds of waxy leaf bearing fewer vapor pressure. In addition, these leaf compounds provide part of the structural components of plants such as cell composition or membrane (Marriott *et al.*, 2001).

The volatile compounds are present in the steam volatile oil, which transfer the pleasant smell (described as camphoraceous, sweet, citrus-like or warm). Since fresh type is more worthy for both aroma and pungency of aroma compounds, these materials contribute a bit to the flavour impact (Bartley & Jacobs, 2000). The smell depends on the concentration and chemical formula of the aroma; while the chemo-physical properties of the aroma itself are responsible for odourants volatility. On the other hand, the vital role is played by molecular weight in which the lesser liberation rate of volatiles is corresponded to the higher molecular weight. Therefore, compounds with lower molecular weights can easily diffuse into matrix more than others can. In addition, it is supposed that intense effects on the strength and the type of interactions with the matrix are induced by the nature and the position of functional groups in the structure of odourants. However, some exceptions are including functional groups nature; functional groups number, double bonds, molecular weight and the polarity of the compound have been investigated and reported. In general, both matrix composition and polarity of the substance control the ability of substances to be broadcasted by the matrix and released across environment (Biniecka & Caroli, 2011).

It would be supposed that the activity of extracted volatile oils is relate to the respective composition of which, their functional groups, the possibility of synergistic interactions between components, the proportions in which these oils are present and the chemical configuration of forming components of them (Delaquis *et al.*, 2002; Dorman & Deans, 2000; Kowalski *et al.*, 2015).

The chemical composition of EOs depends on the place of origin, climatic conditions, and plant species (Martinez *et al.*, 2006). In this concept, it has been presented that any change in EOs structures exhibits different activities. The notable differentiation of EOs composition from various parts of a specific plant has also been proven (Arras & Grella, 1992; Cosentino *et al.*, 1999; Marotti *et al.*, 1994; McGimpsey *et al.*, 1994; Marino *et al.*, 1999; Juliano *et al.*, 2000; Faleiro *et al.*, 2002; Lis-Balchin *et al.*, 1999; Pragadheesh *et al.*, 2013; Pragadheesh *et al.*, 2015; Tranchida *et al.*, 2013; Lermen *et al.*, 2015).

Table 4.1 Some main components of essential oils.

Essential oil	Plant part	Botanical source	Main components	% of total
Angelica	Roots	<i>Angelica archangelica L.</i>	α -pinene	24.7
			δ -3-Carene	10.5
			α -phellandrenetmyrcene	10.8
			Limonene	12.9
			β -phellandrene	10.4
			<i>p</i> -Cymene	7.7
Bergamot	Fruits	<i>Citrus bergamia</i> <i>Risso et poit</i>	β -pinene	7.7
			Limonene + β -phellandrene	39.4
			γ -Terpinene	8.6
			Linalool	11.1
			Linalyl acetate	28.0
Cinnamon	Inner bark	<i>Cinnamomun</i> <i>Zeylanicum Blu.</i>	(<i>E</i>)-cinnamaldehyde	77.1
			Eugenol	7.2
			<i>p</i> -cymene	6.1
			Linalool	72.0
Dill (Indian)	Seeds	<i>AnethumsowaRoxb</i>	Limonene	50.9
			Trans-Dihydrocarvone	10.4
			Carvone	20.3
			Dillapiole	36.6
Eucalyptus	Leaves	<i>Eucalyptus Citriodora</i>	Citronellal	72.8
			Citronellol	14.5
Ginger	Roots	<i>Zingiber officinal</i> <i>Rosc.</i>	Camphene	14.1
			Neral	4.9
			Geranial + bornyl acetate	8.1
			β -Bisabolene	22.1
			ar-Curcumene	14.5
			β -Eudesmol	5.4
Juniper	Berries	<i>Juniperus commuunis L.</i>	α -pinene	33.7
			Sabinene	27.6
			Myrcene	5.5
Orange	Peel	<i>Citrus sinensis L. Osbeck</i>	Limonene	91.5
Pepper	Fruits	<i>Piper nigrum L.</i>	α -pinene	9.0
			β -pinene	10.4
			Sabinene	19.4
			δ -3-Carene	5.4
			Limonene	17.5
			β -Caryophyllene	14.7
Rosemary	Whole plant	<i>Rosemarinus officinalis L.</i>	α -pinene	7.4
			β -pinene	5.0
			1,8-Cineole	43.6
			Camphor	12.3
Tea tree	Branches	<i>Melaleuca alternifolia L.</i>	α -Terpinene	10.4
			1,8-Cineole	5.1
			Terpinene-4-ol	40.1
			γ -Terpinene	23.0

There have been observed differences in the biological and ethnomedicinal activities including metabolism, carminative, tonic, digestive, antispasmodic, antioxidant and anti-inflammatory. Other applications are summarised into antimicrobial and antibacterial activities, antiviral activities, expectorant properties for the treatment of cold and physical properties such as selective interaction to specific compounds of EOs which suggest that the chemical components of the oils are affected by susceptibility of various microorganisms (Martinez *et al.*, 2006; Nikolic *et al.*, 2014; Pragadheesh *et al.*, 2015; Li *et al.*, 2015; Nickavar *et al.*, 2005; Ghasemi, 2009; Pirbalouti *et al.*, 2013; Engel, 2001; Champagne & Boutry, 2016; Bhatti *et al.*, 2014).

Moreover, there have been reported different kinds of medicinal organic compounds, including sterols and triterpenes (Manayi *et al.*, 2016; Adesida *et al.*, 1972), alkaloids (Silva *et al.*, 2016; Foussard-Blampin *et al.*, 1967; Willamman & Li, 1970; Mensah *et al.*, 1988; Cosentino *et al.*, 1999), tannins (Salini & Pandian, 2015), and flavonoids (Martín-Rodríguez *et al.*, 2015).

EOs are composed of 20–60 known components (Senatore, 1996; Russo *et al.*, 1998) which major components can constitute from trace to nearly 85% of the EO (Senatore, 1996; Bauer *et al.*, 2001). In generally, these major components determine the biological properties of the essential oils (Pavela, 2015).

The main groups of EOs are divided to two groups: (i) aromatic and aliphatic compounds, and (ii) hydrocarbon terpenes (isoprenes) and terpenoids (isoprenoids). As mentioned previously, many of EOs (cade oil, anise oil, cinnamon oil, capsicum oil, bud oil, clove, garlic oil, dill oil, oregano oil, ginger oil and tea tree oil) and their main compounds (benzyl salicylate, anethol, carvone, carvacrol, eugenol and cinnamaldehyde) have remarkable properties (Cosentino *et al.*, 1999). Amongst all of these properties, Table 4.2 shows several EOs bearing components that proposed antibacterial properties. Also, Figure 4.1 illustrates some chemical structures have been observed in EOs (Burt, 2004; Benchaar *et al.*, 2008).

The main groups of EOs are divided to two groups: (i) aromatic and aliphatic compounds, and (ii) hydrocarbon terpenes (isoprenes) and terpenoids (isoprenoids) (Cosentino *et al.*, 1999). The main terpenes are the monoterpenes and sesquiterpenes. In addition, hemiterpenes, diterpenes, triterpenes and tetraterpenes are exist. Terpenoids are biochemically modified terpenes in which enzymes can add oxygen molecules and remove or move a methyl group. A terpene-containing oxygen is called a terpenoid. On the other hand, terpenoids also called isoprenoids which are oxygenated family of terpenes as hydrocarbon such as phenols, alcohols, ketones, aldehydes, acids, esters and ethers (Bakkali *et al.*, 2008). Terpenoids comprise overlay both oxygenated monoterpenes and sesquiterpenes or sesquiterpenoids (El Asbahani *et al.*, 2015).

Almost over of terpenes are synthesised through branched five-carbon (isoprene units) condensation. The combination of isoprene units (5-carbon-base (C₅)) will form a group of secondary metabolites entitle terpenes. Isoprene units (2-methyl-1,3-butadiene) are the essential building blocks of terpenes that are linked in a head-to-tail model. Different combinations of the isoprene units originate structurally and functionally different classes of terpenes (Rubio *et al.*, 2013). Monoterpenes, sesquiterpenes and diterpenes may have a diversity of aliphatic hydrocarbons (especially low molecular weights such as ramified, linear, saturated, and unsaturated), lactones, alcohols, acids, acyclic esters or aldehydes, coumarins, compounds bearing nitrogen and sulphur and homologues of phenylpropanoids (Dorman, 1999).

Table 4.2 Major components of selected EOs that exhibit antibacterial properties.

Common name of EO	Latin name of plant source	Major Components	Approximate % composition	References
Cilantro	<i>Coriandrum sativum</i> (immature leaves)	Linalool	26%	(Delaquis <i>et al.</i> , 2002)
		E-z-decanal	20%	
Cariander	<i>Coriandrum sativum</i> (seeds)	Linalool	70%	(Delaquis <i>et al.</i> , 2002)
		E-z-decanal	0%	
Cinnamon	<i>Cinnamomum zelandicum</i>	Trans-cinnamaldehyde	65%	(Lis-Balchin <i>et al.</i> , 1999)
Oregano	<i>Origanum vulgare</i>	Carvacrol	Trace-80%	(Lawrence, 1984a; Charai <i>et al.</i> , 1996; Prudent <i>et al.</i> , 1995; Sivropoulou <i>et al.</i> , 1996; Kokkini <i>et al.</i> , 1997, Russo <i>et al.</i> , 1998; Daferera <i>et al.</i> , 2000; Demetzos & Perdetzoglou, 2001; Marino <i>et al.</i> , 2001)
		Thymol	Trace-64%	
		γ -Terpinene	2-52%	
		p-Cymene	Trace-52%	
Rosemary	<i>Rosmarinus officinalis</i>	α -pinene	2-25%	(Daferera <i>et al.</i> , 2000; Daferera <i>et al.</i> , 2003; Pintore <i>et al.</i> , 2002)
		Bornyl acetate	0-17%	
		Camphor	2-14%	
		1,8-cineole	3-89%	
Sage	<i>Salvia officinalis</i> L.	Camphor	6-15%	(Marino <i>et al.</i> , 2001)
		α -pinene	4-5%	
		β -pinene	2-10%	
		1,8-cineole	6-14%	
		α -thujone	20-42%	
Clove (bud)	<i>Syzygium aromaticum</i>	Eugenol	75-85%	(Bauer <i>et al.</i> , 2001)
		Eugenyl acetate	8-15%	
Thyme	<i>Thymus vulgavis</i>	Thymol	10-64%	(Lens-Lisbonne <i>et al.</i> , 1987; McGimpsey <i>et al.</i> , 1994; Cosentino <i>et al.</i> , 1999; Marino <i>et al.</i> , 1999; Daferera <i>et al.</i> , 2000; Juliano <i>et al.</i> , 2000)
		Carvacrol	2-11%	
		γ -Terpinene	2-31%	
		β -Cymene	10-56%	

Over previous classification, EOs can be derived to terpene-less and sesquiterpene-less deprived or corrected of a substance by partial removal like methyleugenol and furocoumarines in rose and citrus oil respectively (European Pharmacopoeia, 2008).

The biological activity and characteristic aroma of EOs are generally induced from monoterpenes, sesquiterpenes and oxygenated derivatives of them, which are lipophilic structures (Brusotti *et al.*, 2014). In medicinal and aromatic plants, terpenes and terpenoids are responsible for the medicinal, fragrant and culinary applications (Dorman, 1999). Amongst natural products, terpenoids are the largest group and there are over 40,000 different terpenoids have been recognised which comprise 90% of EOs. Terpenes

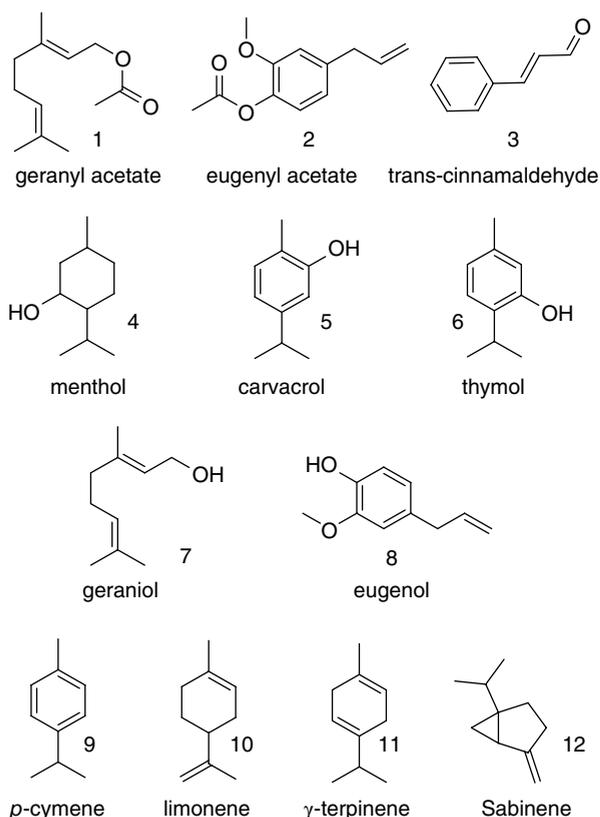


Figure 4.1 The chemical structures of some EOs.

have absorbed commercial attentions due to their remarkable features including prevention of several diseases like cancer and their insecticide activities. They have useful properties in agricultural produce such as sprouting inhibitors in various potatoes, and are building blocks for the synthesising of worthy molecules (Mashwani *et al.*, 2016; Champagne & Boutry, 2016).

Both of biosynthetic studies and targeted breeding are based on chemical diversity (Franz & Novak, 2010). It was observed that the methyl erythriol phosphate and mevalonic acid were applied for the biosynthesis of monoterpenes and sesquiterpenes, respectively (Rodriguez-Concepcion & Boronat, 2002). Recently, the biosynthesis of EOs (especially by enzymes) has been investigated by researchers to improved oil composition and yield (Wildung & Croteau, 2005).

4.2 Chemical Composition of Essential Oils

4.2.1 Terpenes and Terpenoids

As mentioned, the combination of isoprene units (5-carbon-base (C₅) or 2-methyl-1,3-butadiene) to form terpenes as secondary metabolites followed head-to-tail model (Rubio

et al., 2013). Different isoprene combinations originate functionally and structurally the various groups of terpenes (Rubio *et al.*, 2013). The classification of which is according to isoprene units number by structural formula $(C_5H_8)_n$, which n is the number of linked isoprene units, for example, two isoprene units form monoterpenes (two C_5 groups) with molecular formula $C_{10}H_{16}$ (Dorman & Deans, 2000). Other conjugations produce different compounds such as diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}) and also alternative hemiterpenes (C_5) and sesquiterpenes (C_{15}). Monoterpenes, diterpenes, and sesquiterpenes are the main groups of terpenes found in spices and herbs have notable biological activities such as antimicrobial effects on different pathogens like *Candida* species (Alves *et al.*, 2013; Rubio *et al.*, 2013; Saleem *et al.*, 2010).

Terpenes can be found widely in nature. They are inexpensive components and are available easily in large scale. Monoterpenes in plants — as attractants for and pollinators antifungal defences — have ecologically disincentive roles against feeding by herbivores (Langenheim, 1994). In mammals, they are applied as regulators of enzymatic processes, in metabolic pathways, and stabilising cell membranes (De Carvalho & Da Fonseca, 2006). Terpenes may also be useful for chlorinated mediums in practical tasks such as aircraft parts cleaning, cleaning of electronic components and cables, and degreasing of metal (Brown *et al.*, 1992). The industrial monoterpenes usages as ozone-depleting chlorofluorocarbons alternates is also under studying (Kirchner, 1994).

Monoterpenes are the most delegate structures covering a wide range of monocyclic, bicyclic and acyclic components and organic functional groups including hydrocarbons such as camphene, *p*-cimene and myrcene, and alcohols such as borneol menthol and linalool. Other functional groups have been introduced like aldehydes such as citronellal and geranial; ketones such as camphor, carvone and pulegone; esters such as citronellyl acetate, linalyl acetate and menthyl; ethers such as 1,8-cineole and menthofurane; peroxides such as ascaridole; phenols such as thymol and carvacrol. Many plants like cannabis, orange, rosemary, angelica, bay leaves, thyme, celery, parsley, ylang-ylang, laurel, hops, mugwort, mint, tea tree, wormwood, bergamot and sweet basil contain these compounds (Pavela, 2015).

As discussed in previous paragraphs, having assembled three isoprene units (C_{15}), sesquiterpenes go on to form. The chain extension increases the number of cyclisations causes the production of a wide diversity of structures. In comparison to monoterpenes, sesquiterpenes have the same structure: Hydrocarbons: β -bisabolene, β -caryophyllene, azulene, cadinenes, logifolene, elemenes, curcumenes, zingiberene and farnesenes; Alcohols: cedrol, bisabol, β -nerolidol, β -santalol, farnesol, patchoulol, carotol and viridiflorol; Ketones: nootkatone, germacrone, cis- β -vetinone, longipinan-2,7-dione and turmerones; Epoxide: caryophyllene oxide and humulene epoxides. Examples of plants containing these compounds are lemon, bergamot, mint, angelica, coriander, celery, eucalyptus, caraway, citronella, juniper, geranium, lavender, lavandin, mandarin, lemongrass, peppermint, orange, pine, petitgrain, sage, rosemary and thyme (Bakkali *et al.*, 2008; Banthorpe, 1991).

In these types, the basic five-carbon units assembled as same as sequential combining of terpenes helps scientists to investigate the familiar orders of C_{10} (mono-), C_{15} (sesqui-), C_{20} (di-), C_{30} (tri-), C_{40} (tetra-) and $C > 40$ (poly-) terpenoids (Table 4.3 and Figure 4.2) (Marriotta *et al.*, 2001). They are characteristically observed in plants as the mixtures of compounds in which five-carbon groups can be found in different types. The 'lower terpenoids' refers commonly to C_{10} and C_{15} compounds and they are usually

Table 4.3 Terpene nomenclature for isoprenes.

Carbon atoms	Isoprene units	Nomenclature
40	8	Tetraterpenes
30	6	Triterpenes
25	5	Sesterterpenes
20	4	Diterpenes
15	3	Sesquiterpenes
10	2	Monoterpenes

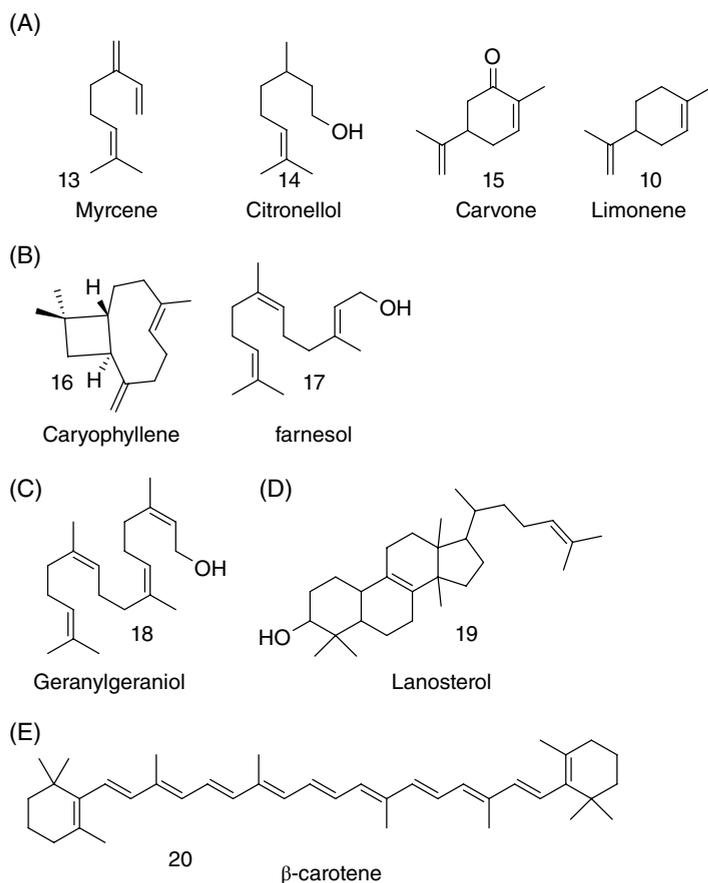


Figure 4.2 Structures of selected typical terpenes. (A) Monoterpenes: 13 = myrcene, 14 = Citronellol, 15 = carvone, 10 = limonene; (B) sesquiterpenes: 16 = caryophyllene, 17 = farnesol; (C) diterpene: 18 = granlygeraniol; (D) triterpene: 19 = lanosterol; (E) tetraterpene: 20 = β -carotene.

called 'essential oils' when they occur together. On the other hand, 'higher terpenoids' are the concept mentions both compounds bearing 20 and more carbons. As the latest group, the term 'resin' is used when the nonvolatile terpenoids (diterpenoids or triterpenoids) combine to volatile ones (monoterpenoids and/or sesquiterpenoids) (Langenheim, 1994).

Although terpenoids have been recognised as lipophilic compounds, there are many reports have shown that these conclusions must be considered carefully more than we used to. For example, some oxygenated volatile monoterpenoids that previously thought to be lipophilic molecules, at biologically active concentrations are water soluble and the ecological implications of which have also been investigated. These water-soluble monoterpenoids are amongst to those terpenoids occur as glycosides. For example, saponins and cardenolides as triterpenoid and the iridoids monoterpenes have glucosidic binds. Some terpenoids are volatile (mono- and sesquiterpenes), whereas some others are not (di- and triterpenes,). In fact, the volatility is the most applicable worthy feature for monoterpenoids (Langenheim, 1990). During last centuries, flora bearing terpenoids and the oxygenated derivatives of them have been consumed as flavours and fragrances (De Carvalho & Da Fonseca, 2006).

4.2.2 Aromatic Compounds

In generally, aromatic compounds occurs less customarily than terpenes. There are many plant sources comprising these compounds including anise, nutmeg, clove, parsley, fennel, star anise, sassafras, tarragon and some other botanical groups (Lamiaceae, Apiaceae, Rutaceae, Myrtaceae) (Grayson, 2000; Pavela, 2015). Similar to phenylpropane, these compounds are usually found less than the terpenes. The biosynthetic approaches concerning terpenes and phenylpropanic derivatives are generally separated in plants but in some cases, they can be produced through specific pathways (Bakkali *et al.*, 2008).

The biosynthetic approaches of phenylpropanoids and terpenes derivatives are generally extracted in plants but in some cases, it may be coexisted with the major method taking over (Grayson, 2000; Pavela, 2015; Bakkali *et al.*, 2008).

Aromatic compounds have the same applications to those of monoterpenoids and sesquiterpenoids. They contain alcohols (cinnamic alcohol); aldehydes (cinnamaldehyde); phenols (chavicol, eugenol); methoxy compounds (methyleugenol, elemicine, estragole, anethole), and methylene dioxy derivatives (safrole, myristine, apiole) (Figure 4.3) (Grayson, 2000).

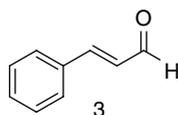
4.3 Synthesis and Biosynthesis of Essential Oils

The process of EOs synthesis is done in the plastids and cytoplasm of plant cells via methyl-D-erythritol-4-phosphate (MEP), mevalonic acid and malonic acid pathways (Pavela, 2015). Complex secretory structures are as container for the synthesising and storing of EOs compounds, namely resin ducts, secretory cavities and glandular trichomes, and as droplets of fluid, they are obtained from the fruits, flowers, bark, roots, leaves and stem of plants (Fahn, 2000). These mixtures are complex natural materials bearing around 20–60 compounds at completely varies concentrations. In comparison to components that are found in trace amounts, these molecules can be identified by two or three main components at high concentrations (20–70%) and generally exhibit the biological activity of EOs (Pavela, 2015).

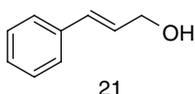
The chemicals prepared by nature have been categorised into two groups: (i) the primary metabolites with four subgroups including carbohydrates, proteins, lipids and nucleic acids that have minimal participation to EOs although the degradation of one of

Aromatic Compounds**Aldehyde**

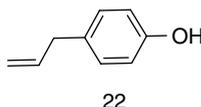
Cinnamaldehyde

**Alcohol**

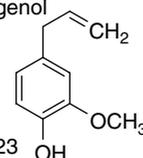
Cinnamyl alcohol

**Phenol**

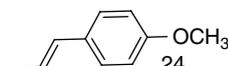
Chavicol

**Phenol**

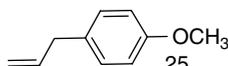
Eugenol

**Methoxy derivatives**

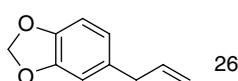
Anethol

**Methoxy derivatives**

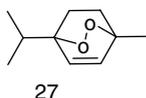
Estragole

**Methylene dioxy compound**

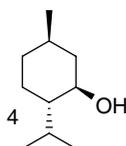
Safrole

**Terpenoides (Isoprenoides)**

Ascaridole



Menthol

**Figure 4.3** The structure of some aromatic compounds.

these groups can produce some EO components, lipids being the most significant. They are universal across animal and plant families and vocalise life's basic building blocks; (ii) the secondary metabolites which happen in specific ones and not others. They are also ordered into alkaloids, polyketides, shikimates and terpenoids. The latest one is the most vital as far as EOs are concerned and the second types are shikimates (Mann *et al.*, 1994).

Primary metabolites have the ability to form natural products or plant secondary metabolites (PSMs) bearing various physiological activities. The essential effect of secondary metabolites (SMs) in plants is to make strong interactions between the environment and plants for their fitness and survival that causes PSMs to be as vital as primary metabolites. However, PSMs have no instant effects in plants' survival, but they have customary long-term effects. Over 100,000 SMs have been produced by the plant kingdom that are limited to taxonomic groups. PSMs are biosynthetically categorised into three groups involving terpenes (isoprenoids), nitrogen-containing compounds (cyanogenic glycosides, glucosinolates and alkaloids), and phenolic compounds (flavonoids and phenylpropanoids) (Figure 4.4) (Verma & Shukla, 2015).

Green plants can convert water and carbon dioxide into glucose through photosynthesis and cleaving of glucose produces key building blocks for the shikimates of natural products entitled phosphoenolpyruvate. Two-carbon units of acetate are composed by the decarboxylation of which and coenzyme-A can esterify this to compose acetyl-CoA; whereas lipids and polyketides are given by self-condensation of these species. The key starting material for the synthesis of terpenoids is mevalonic acid that is another target for acetyl-CoA which is applied as a starting point. There are other possibilities for the synthesis of which including the use of methylerythritol phosphate (MEP),

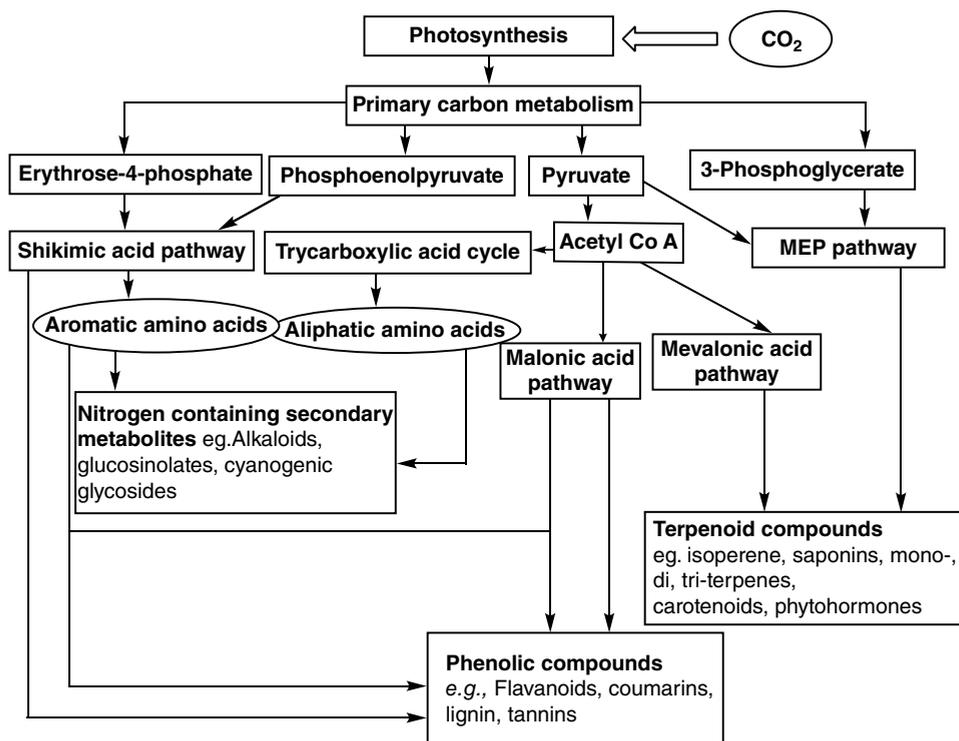


Figure 4.4 A general sketch of biosynthetic pathway of secondary metabolites in plants.

1-deoxyxylulose-1-phosphate synthase (DXS), mevalonate-dependent (MVA) pathway and 1-deoxy-d-xylulose-5-phosphate (DXP). In all of these reactions and natural chemistry, nature uses the reactions as same as whom are done by chemists (Sell, 2003; Opitz *et al.*, 2014; Mendoza-Poudereuxa *et al.*, 2014). Anyway, nature's reactions use enzymes (globular proteins in which the reacting species are hold together by an active site) as catalyst. Thus, they tend to be more selective and faster than which do not use enzyme. These organisations lower the reaction activation energy in active site and direct their stereochemical courses (Baser & Buchbauer, 2010).

Cofactors are vital for a lot of enzymes and are required as energy or reagents providers. Coenzyme-A is a thiol and is utilised for the synthesising thioester derivatives with carboxylic acids. An essential role of adenosine triphosphate (ATP) in biosynthesis is phosphorylation or making phosphate esters of alcohols. Adenosine diphosphate (ADP) and phosphate ester are given by adding of one ATP phosphate group to the alcohol. Another biosynthetically important group of cofactors contains pairs including DPN/DPNH, TPN/TPNH and NADP/NADPH. These cofactors comprise an *N*-alkylated pyridine ring and one part of each pair has *N*-alkyl-1,4-dihydropyridine and the other one has an *N*-alkylated pyridinium salt. Redox reagents can be happened when two structures in each pair would have capabled to be interconverted by gaining or losing a hydride anion. Although the reactive part of all cofactors is a small section of them, the most considerable observation is that the bulk of the molecule is responsible to the molecular recognition. The cofactor joins

into enzyme active site through recognition. In comparison to substrate, cofactors are holed in the optimum spatial configuration during this occurrence (Baser & Buchbauer, 2015).

4.3.1 Terpenes and Terpenoids

Terpenes are a subcategory of EOs and their synthetic pattern is the head-to tail joining of the isoprene moieties (Marriotta *et al.*, 2001). As demonstrated in Figure 4.5, the conjugating of isoprene units is usually in one possibility entitled head-to-tail type coupling. The branched end of chain is related to the molecule head and the other end is considered as the tail (Baser & Buchbauer, 2010).

The biosynthesis of isoprenoids and prennylipids in plants is occurred through two individual ways: (i) the cytosolic classical acetate/mevalonate method for triterpenoids biosynthesis, sesquiterpenes, sterols; and (ii) non-mevalonate 1-deoxy-D-xylulose-5-phosphate (DOXP) approach for plastidic isoprenoids biosynthesis, such as phytol (a side-chain of chlorophylls), carotenoids, plastoquinone-9, mono- and diterpenes and isoprene. Both of them give active C₅-unit isopentenyl diphosphate (IPP) as the starting materials from which. All other isoprenoids are composed through the head-to-tail addition approach (Lichtenthaler, 1999).

Isoprene (C₅H₈) or 2-methyl-1,3-butadien is the most rudimentary hemiterpene which is exhaled by a lot of plants per year all over the world (Rasmussen and Khalil, 1988; Zimmermann, 1979; Dignon & Logan, 1990). Glyceraldehyde-3-phosphate (GA-3-P) and pyruvate (hydroxyethyl-thiamine) are the starting matters of the 1-deoxy-D-xylulose-5-phosphate (DOXP) process for the synthesis of isoprene. In the reaction catalyzed by transketolase and dependent upon thiamin, C₂-units that obtained from pyruvate are transferred to GA-3-P, whereby DOXPs are produced (Figure 4.6). The suitable 1-deoxy-D-xylulose (DOX) incorporation into plastidic isoprenoid is an extra proof for this starting step (Figure 4.6). As final evidence for DOXP-synthase as an initial step, it was exhibited that both of a bacterial DOXP-synthase of *Escherichia coli* and a plant DOXP-synthase of *Mentha*, overexpressed in *E. coli*, form DOXP from pyruvate and GA-3-P (Lichtenthaler, 1999).

The IPP process commences from GA-3-P as a benign intermediate within the photosynthetically reduction of carbon and pyruvate, which is generated in the plastid from 3-phosphoglyceric acid. As illustrated in Figure 4.7, the DOXP route transforms carotenoids, isoprene, phytol, mono- and diterpenoids, and the non α -prenyl chain of plastoquinone-9 (Lichtenthaler *et al.*, 1997; Lichtenthaler *et al.*, 1997a; Lichtenthaler *et al.*, 1997b; Disch *et al.*, 1998; Arigoni *et al.*, 1997).

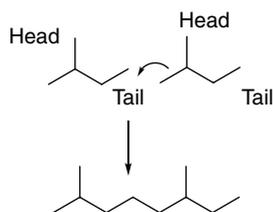


Figure 4.5 The head-to-tail method of two isoprene coupling.

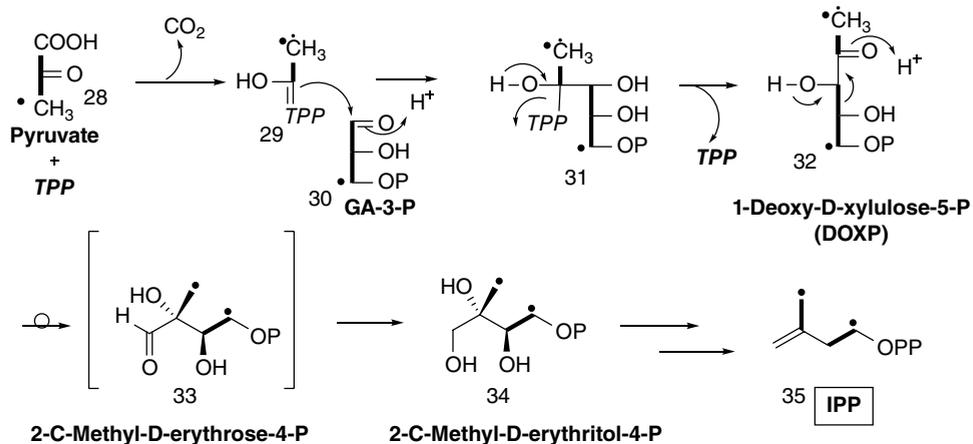


Figure 4.6 Systematically process and proposal possible intermediates in the thiamin (TPP)-dependent biosynthesis of IPP from GA-3-P and pyruvate.

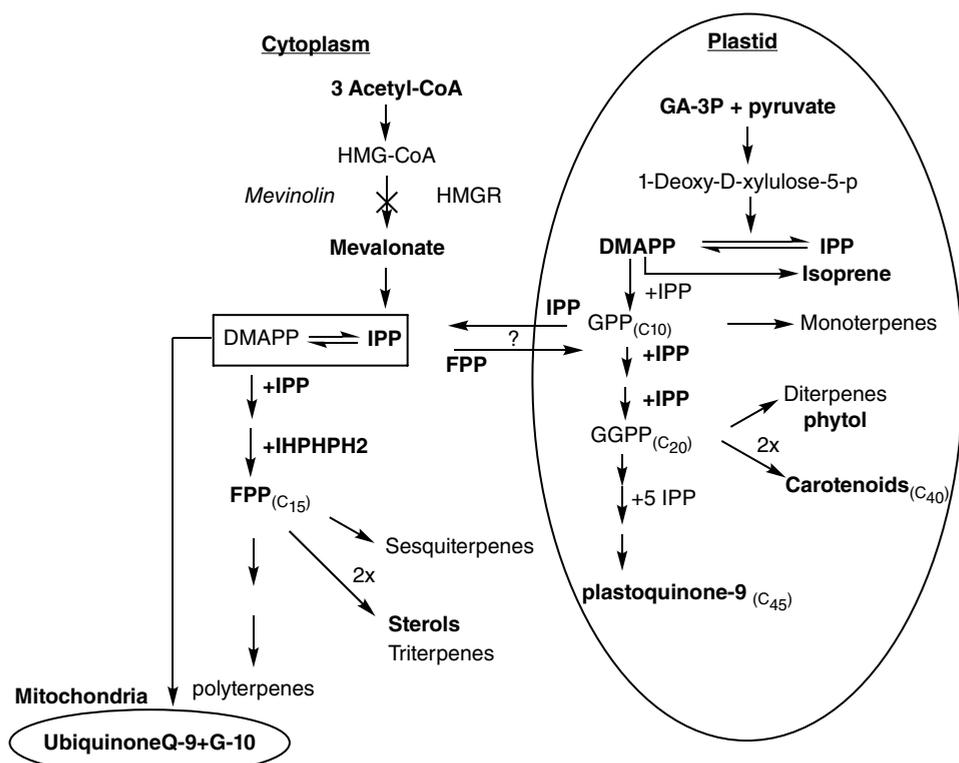


Figure 4.7 The proposed compartmentation of IPP and the biosynthesis of isoprenoid in higher plants between plastids (DOXP process) and cytosol (acetate/MVA process). Source: Lichtenthaler (1997). Reproduced with permission of Springer.

Isoprenes are composed light-dependent (Sharkey & Loreto, 1993) from dimethylallyl pyrophosphate (DMAPP) (36) by an isoprene synthase, which DMAPP is in equilibrium with IPP (Figure 4.8) (Wildermuth & Fall, 1996; Schnitzler *et al.*, 1996; Silver & Fall, 1995; Kuzma & Fall, 1993; Zeidler *et al.*, 1997).

About 50 hemiterpenes are known (Figure 4.8). 2-methyl-1,3-butadiene (isoprene) (37) is non-natural but 3-methyl-2-buten-1-ol (prenol) (38) situates in the oil of hops from *Humulus lupulus* (Cannabaceae) and in ylang-ylang oil captured from chosen flora of *Cananga tree Canangaodorata* (Annonaceae). Biosynthesis of terpenes is from isopentenyl diphosphate (isopentenyl pyrophosphate) (IPP) (39), lysergic acid as derived hemiterpenoid from the amino acid tryptophane and diphosphates of 2-carboxy-1-buten-4-ol. (S)-(-)-3-Methyl-3-buten-2-ol (40) is observed in hops essential oils, oranges, and grapefruit. Flavour of blackcurrant *Ribesnigrum* (Saxifragaceae) is from 4-Methoxy-2-methyl-2-butanthiol (41) (Figure 4.9). The acid part of many natural esters (*e.g.*, ester alkaloids) are tiglic acid, its regioisomers angelic and isovaleric acid as well as 3-methyl-2-butenic acid (Figure 4.10) (Breitmaier, 2006).

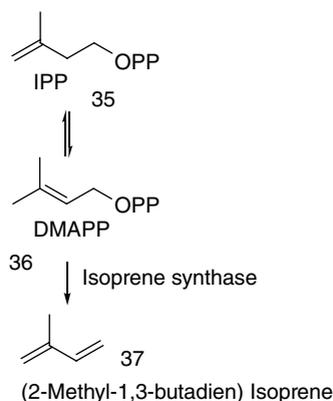


Figure 4.8 Biosynthesis of isoprene from IPP and DMAPP via the isoprene synthase.

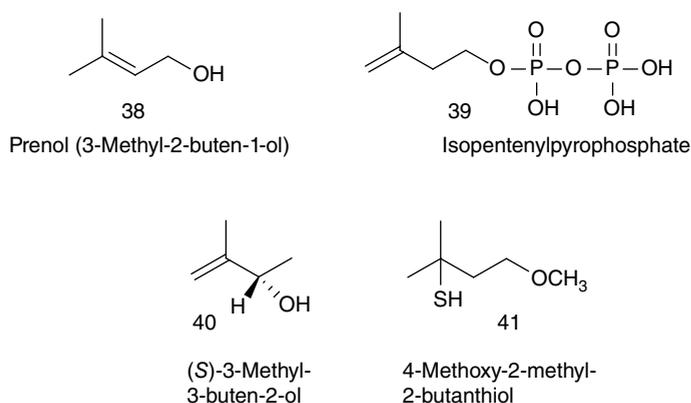


Figure 4.9 The chemical structures of Prenol (3-Methyl-2-buten-1-ol), Isopentenylpyrophosphate, (S)-3-Methyl-3-buten-2-ol and 4-Methoxy-2-methyl-2-butanthiol.

Enzymes have protein structure and uses as biocatalysts for a wide variety of chemical reactions. In general, enzymes can catalyze the transformation of macromolecules to produce novel molecules, besides for growth, repair, and maintenance of cells. There have been many reactions done by living cells where complex systems are there and the biocatalysts that enable such chemical reactions to happen are called enzymes (Eslahi *et al.*, 2014; Tomke & Rathod, 2016; Cao *et al.*, 2016; Royvaran *et al.*, 2016). In the biosynthesis of monoterpenes, the committed step is catalyzed by an enzymatic reaction using monoterpene synthases, which causes the conversion of geranyl diphosphate (GPP) to the parent moieties of different monoterpenes. These enzymes are suitable for the engineering of monoterpene metabolism, and are vital for genetically imparting the ability to form monoterpenes on plants which naturally cannot do that or might be confront some limiting (Wise & Croteau, 1999). An enhancement in the yield and changing in the monoterpene features of the targeted plant are certainly carried out by manipulating the expression of genes. Ectopic expression of limonene synthase in cornmint (*Mentha arvensis*) (Diemer *et al.*, 2001) and peppermint (Diemer *et al.*, 2001; Krasnyanski *et al.*, 1999) has been studied to change the composition of monoterpene in both types and yield enhancement. A promising source of commercially important EOs is Peppermint has been attracted many attentions as a benign model system for the investigation of monoterpene biosynthesis (Mc Conkey *et al.*, 2000; Gershenzon *et al.*, 2000).

Several animal species and flora can prepare the P450 cytochrome oxidases involving numerous metabolic progresses. The first step in menthol biosynthesis is the 3-hydroxylation of limonene by P450 (Figure 4.11) (Lupien, 1999; Dudareva *et al.*, 2004).

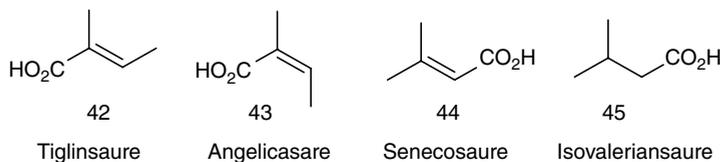


Figure 4.10 The acid part of some natural esters.

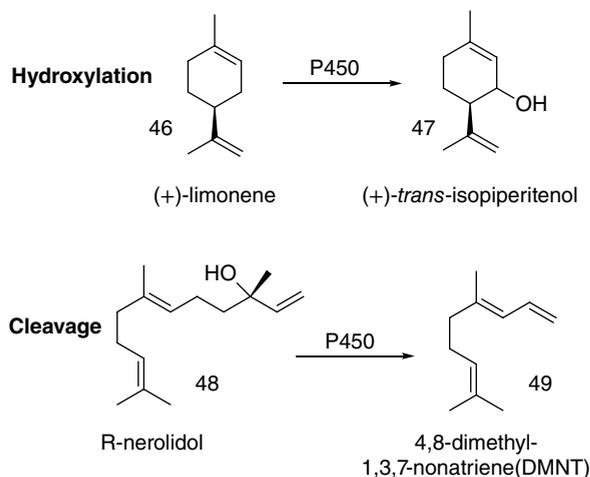


Figure 4.11 A typical modification reaction that causes the biosynthesis of molecules to have increased volatility properties.

The p-menthane monoterpene bio catalytic processes are prevalently occurred in anatomical scaffolds entitled glandular trichomes. The enzyme (-)-limonene synthase can efficiently initiate pathway (Ahkami *et al.*, 2014) (Figure 4.12). Although, (-)-limonene 3-hydroxylase [L3H] catalyses the most common oxygenation reactions in either peppermint or watermint at C3, in the case of spearmint, the catalyzed hydroxylated (-)-limonene should be done at C6 by using (-)-limonene 6-hydroxylase [L6H]. Additional oxidising process of first oxygenated intermediate occurred by (-)-carveol dehydrogenase (spearmint) or (-)-trans-isopiperitenol dehydrogenase (peppermint/watermint). The isomerisation reactions and sequential reduction are catalyzed by (+)-cis-isopulegone isomerase and (-)-trans-isopiperitenone reductase (ISPR) in both watermint and peppermint, lead to the (+)-pulegone formation. The predominant (+)-menthofuran synthase [MFS] catalyzed reaction in watermint is the cyclisation of (+)-pulegone intermediate to (+)-menthofuran, while the more complex peppermint oils are generated through a series of reductions, which can be catalyzed by enzymes (-)-menthone: (-)-menthol reductase (MMR), (+)-pulegone reductase [PR] and (-)-menthone:(+)-neomenthol reductase (Figure 4.11) (Ahkami *et al.*, 2014; Ahkami *et al.*, 2015). Some enzymes applied in this pathways are including: (-)-limonene synthase (LS) and (-)-trans-isopiperitenol dehydrogenase (ISPD). In addition, (-)-limonene 3-hydroxylase (L3H), (-)-limonene 6-hydroxylase (L6H), (+)-cis-isopulegone isomerase (ISPI), (-)-trans-isopiperitenone reductase (ISPR), (+)-menthofuran synthase (MFS) and (+)-pulegone reductase (PR) ((+)-isomenthone-forming and (-)-menthone-forming activities) have been used. Other enzymes are (-)-menthone:(-)-menthol reductase (MMR) ((+)-neoisomenthol-forming activity; MMR1, (-)-menthol-forming activity; MMR2), (-)-menthone:(+)-neomenthol reductase (MNR) (MNR1, (+)-neomenthol-forming activity; MNR2, (+)-isomenthol-forming activity) and (-)-trans-carveol dehydrogenase (CDH).

Menthol is the characteristic and principal monoterpene component which has been specifically accumulated and composed in the peltate glandular trichomes observed on the aerial segment of these plants (Dudareva *et al.*, 2004). (-)-piperitone as the main part of Eucalyptus dives Typus oils can prepare (-)-Menthol. There has been observed a variety of menthols in the hydrogenation using Raney nickel, from which (-)-menthol can be separated by saponification and crystallisation of its chloroacetate (Figure 4.13). The hydrogenations of thymol can also synthesis racemic menthols (Figure 4.14). This leads to a mixture including the four stereoisomeric menthols in different proportions. (-)-Menthol is separated from the other isomers by distillation (Surburg & Panten, 2006).

Metabolic engineering approaches are used to decrease the production of unwanted metabolites in peppermint. Scientists could manipulate the expression of pathway genes leads to menthofuran and pulegone accumulation, while at the same time trying to maximise the production of menthol. The formation of menthofuran from pulegone (Figure 4.11) using antisense methods resulted in remarkable decrease in the concentration of menthofuran in derived oil is due to the reduction of the expression of cytochrome P450 menthofuran synthase (MFS) gene (Mahmoud & Croteau, 2001); suppressing the expression of this gene resulted in virtual elimination of menthofuran from the oil. These data exhibit that metabolic engineering can modify monoterpene biosynthetic processes to optimise the production of benign compounds (Mahmoud & Croteau, 2002; Ahkami *et al.*, 2015; Kitaok *et al.*, 2015).

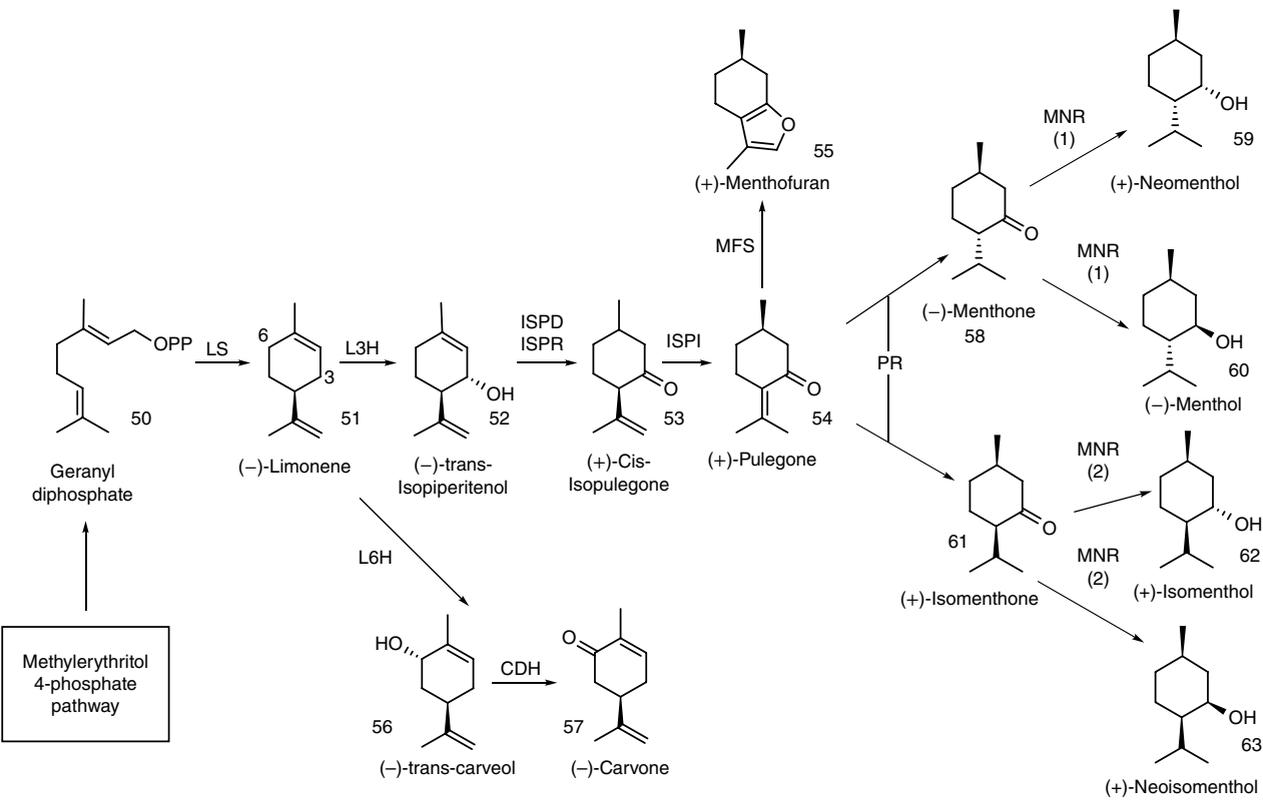


Figure 4.12 Outline biosynthesis of *p*-menthane monoterpene.

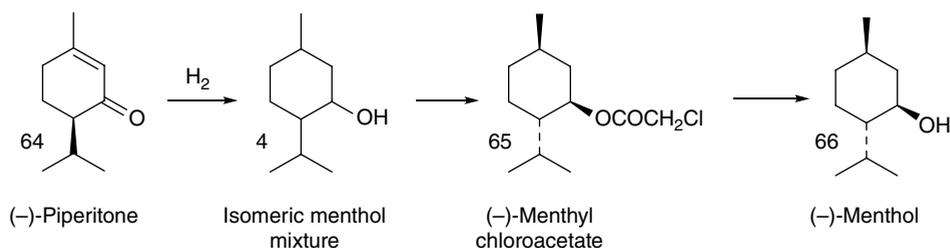


Figure 4.13 The production of menthols by the hydrogenation process using Raney nickel.

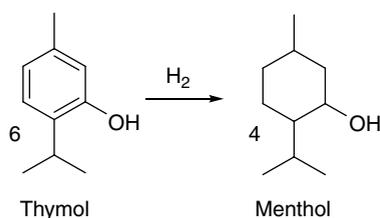


Figure 4.14 The hydrogenations of thymol.

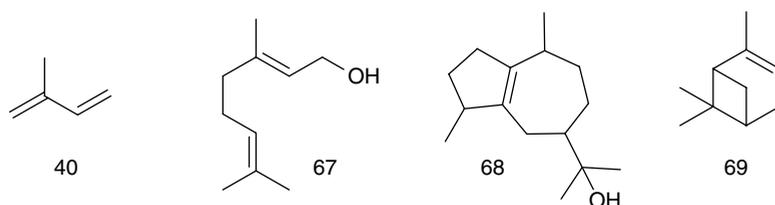


Figure 4.15 Isoprene units in some common terpenoids.

As far as EOs are concerned, the most important groups of natural products are terpenoids. Scientists in older literature introduced them as terpenes but nowadays, this concept referred to monoterpenoids. It has been explained that they are known as substances formed of isoprene (2-methylbutadiene) units. Isoprene is not actually an intermediate in biosynthesis and is not often found in EOs, but the 2-methylbutane template is clearly discernable in terpenoid skeleton. The structure of some terpenoids is shown in Figure 4.15.

The biosynthesis of terpenoids defines this coupling direction (Mann *et al.*, 1994). Mevalonic acid is a very important intermediate (70) is composed from three components of acetyl coenzyme A (Ruzicka & Capato, 1925). Mevalonic acid phosphorylation followed by deletion of tertiary alcohol group and the concomitant decarboxylation of adjacent acid gives isopentenyl pyrophosphate (72) which can be converted to prenyl pyrophosphate by isomerisation (73). As shown in Figure 4.16, 10-carbon units like geranyl pyrophosphate, are prepared by these two 5-carbon units (74) coupling, and more addition of isopentenyl pyrophosphate (72) build 15-, 20-, 25- and larger carbon units (Baser & Buchbauer, 2015).

There have been published several reviews on the biosynthesis of isoprenoids such as monoterpenoids (Bramley, 1997; Dewick, 1999). Geranyl pyrophosphates (74) are as

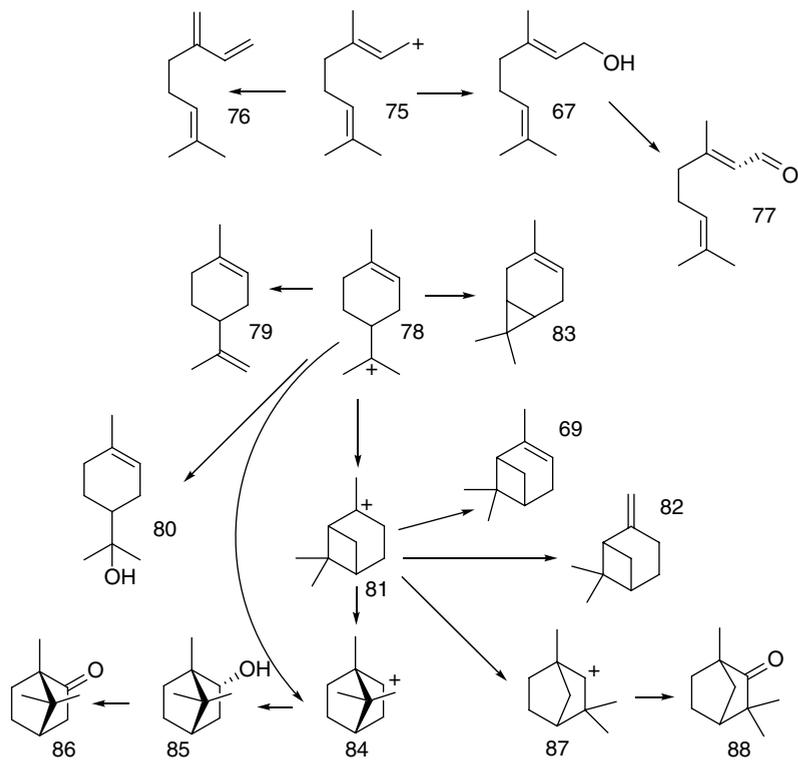


Figure 4.17 Formation of monoterpoid skeletons.

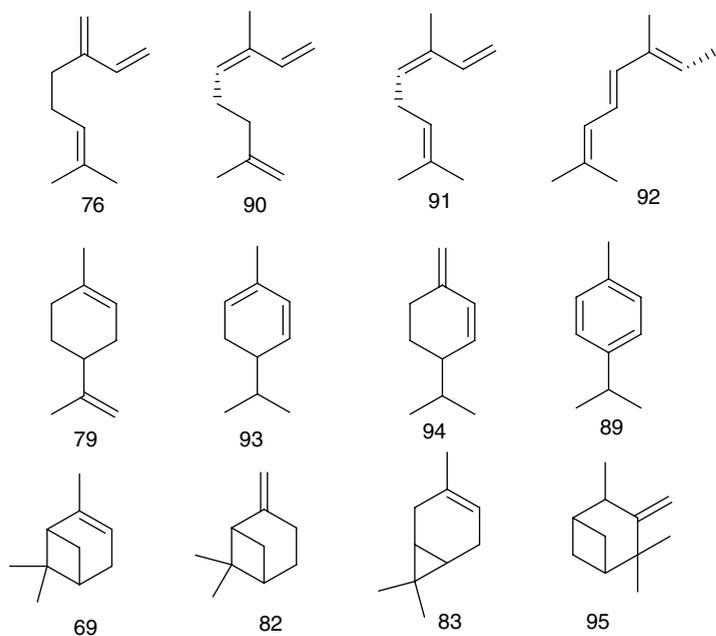


Figure 4.18 Some of usual terpenoid skeleton.

and glyceraldehyde-3-phosphate (Eisenreich *et al.*, 1997; Duvold *et al.*, 1997) and there are reports in the literature based on current monoterpenoids transformations that microorganisms can effect on them (Miyazawa, 1997). It has been investigated the biosynthesis of different ester structures of monoterpenoid using lipase-catalyzed alcoholysis reactions in solvent-free mediums. A great range of yeasts have been studied due to their capacity to biotransform both unsaturated and saturated monoterpenoid ketones (Chatterjee & Bhattacharyya, 1998; Dyk *et al.*, 1998; Hall *et al.*, 2016). Monoterpenoid acetates are useful spectrums of fragrances and flavours were oxidised efficiently by monooxygenase. The selective oxidation of monoterpenoid acetates like sclareolide by this enzyme is given the considerable amount of potential sites for oxidation. This oxidation process is also would be profitable in the field of synthetic chemistry (Hall *et al.*, 2016).

The pharmacology and chemistry of monoterpenoid indole alkaloids has been also reported. The six new kinabalarines (96–101) have been obtained from *Kopsiapauciflora* (Saxton, 1997; Cordell, 1999; Kam *et al.*, 1997), and incarvine D (102), methoxyin carvillateine (103) and the *N*-oxides (104) and (105) have been isolated from *Incarvilleasinensis*. Both of (104) and (105) were given as diastereoisomers mixtures (Figure 4.19). New monoterpenoid alkaloids extracted from *Osmanthus austrocaledonica* include austrodimerine (107) and dihydrojasminine (106) (Figure 4.19) (Chi *et al.*, 1997; Benkrief *et al.*, 1998).

The deglycosylation of 8,10-dihydro-*N*-methylbakankosine (108) that was catalysed by acid proceeds to generate the epimerised glycone (109), but the parent component (110) affords normally the product (111) through a complex rearrangement process has been subjected to graph analysis treatments (Figure 4.20) (Schwartz *et al.*, 1997).

The opisthotonus gland secretion of template mite *Tyrophagus putrescentiae* presents the novel lactone β -acariolide (112). Some pyronene and cyclocitral derivatives that have been explored involve (115–119) from *Gardenia* fruits, and (113) and (114) from *Bupleurum gibraltarium* (Figure 4.21) (Morino *et al.*, 1997; Barrero *et al.*, 1998; Machida *et al.*, 1998).

The regeneration of Pd–Al₂O₃ was applied for catalyzed hydrogenation of dehydrolinalool (120) and the same hydrogenation can also be done over palladium based colloidal catalysts have been composed in poly(styrene)–poly(4-vinylpyridine) micelles (Sulman *et al.*, 1998; Sulman *et al.*, 1999). Tetrahydrofuran (125) has been obtained from geranyl acetate (123) via OsO₄-catalyzed hydroxylation in the presence of NaIO₄ in DMF (Figure 4.22) (Champdore *et al.*, 1998).

Artemisia fragrans produce the peroxide (126) bearing an irregular santolinyl group (Marco *et al.*, 1998) (Figure 4.22). (+)-lasiol (128) is in the mandibular gland secretion of males of the ant *Lasius meridionalis*. There has been reported as same route as those for (±)- and (*R*)-lavandulol (127), and (±)-hotrienol (129) for this compound. It has been synthesised Artemisia ketone (130) through an indium-mediated prenylation of the dimethyl acryloyl pyrazole (131) (Figure 4.23) (Schneider, 1998; Fleming & Lee, 1998; Faure & Piva, 1998; Bohmer *et al.*, 1997; Bryan & Chan, 1997).

The hydroxycineol glycoside compound (132) has been extracted from the fruits of *Torillia japonica* (Figure 4.23) (Kitajima *et al.*, 1998c), and an aqueous extract of *Foeniculum vulgare* presented any stereoisomers of 2-hydroxy-1,8-cineol- β -D-glucopyranoside. Five 1,8-cineol glycosides together as well as new dihydroxycineoles (133–137) have been synthesised with the similar source. The homomonoterpenoid fucisic acid (138) has also been isolated from *Ficus macrocarpa* (Figure 4.24) (Ishikawa *et al.*, 1998b; Li & Kuo, 1998).

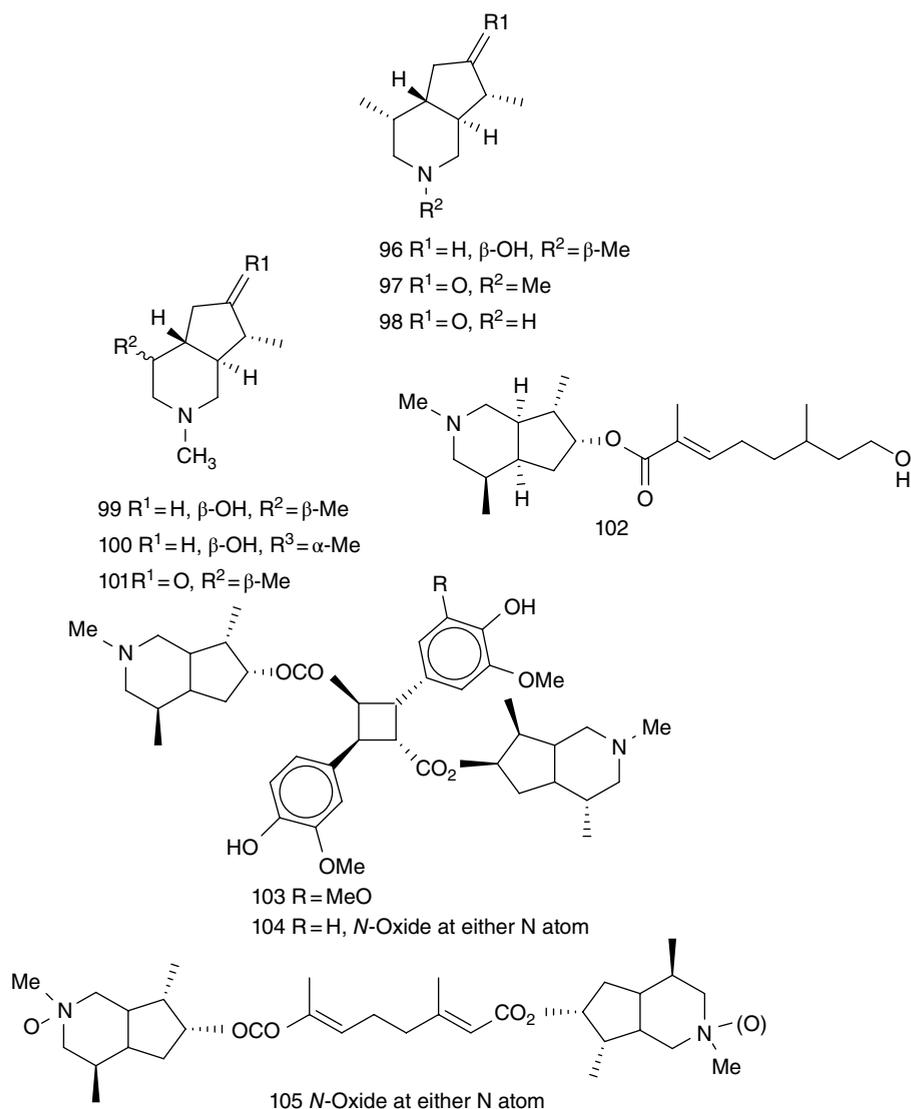


Figure 4.19 The chemistry and structure of some monoterpenoidindole alkaloids.

Figure 4.25 exhibits the structure of some obtained menthanes. They are a group of these molecules and some epoxyacetates such as (139) and (140) have been isolated from *Mentharotundifolia* (Umomoto, 1998), while tetraol (141) has been observed in the fruits of *Cnidiummonnieri* (Kitajima *et al.*, 1998a). The unusual *p*-menthatrienol (143) and (*E*)-5-(1-terpinen-4-olyl)-3-methoxystilbene (142) has been isolated from *Ligulariaintermedia* and *Alpiniakatsumadair* respectively (Ngo & Brown, 1998; Chen *et al.*, 1998). The biosynthesis of carvone (15) and limonene (10) in the fruits of *Carumcarvi* has been studied (Bouwmeester *et al.*, 1998) and when (\pm)-Limonene is metabolised by *Penicilliumdigitatum* (10), affords (*R*)-(+)- α -terpineol (145) (Tan *et al.*, 1998a). When

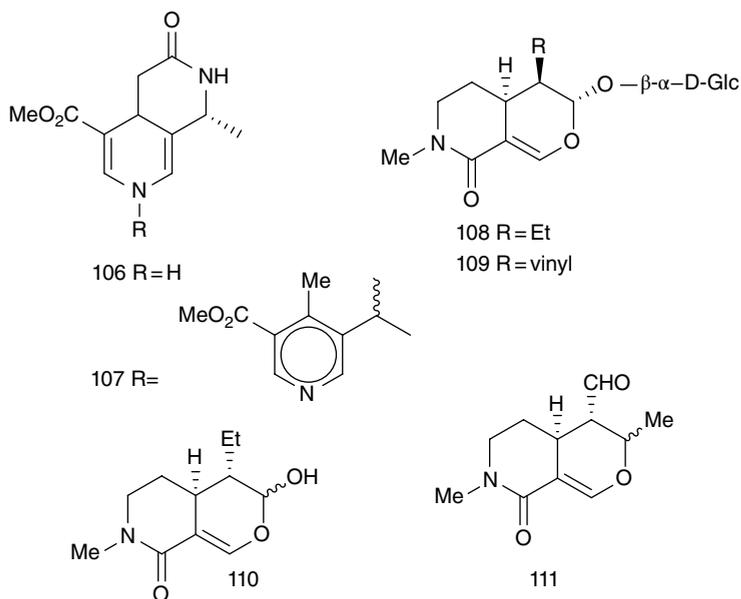


Figure 4.20 Produced component through acid catalyzed deglycosylation reaction of 8,10-dihydro-*N*-methylbakankosine (108).

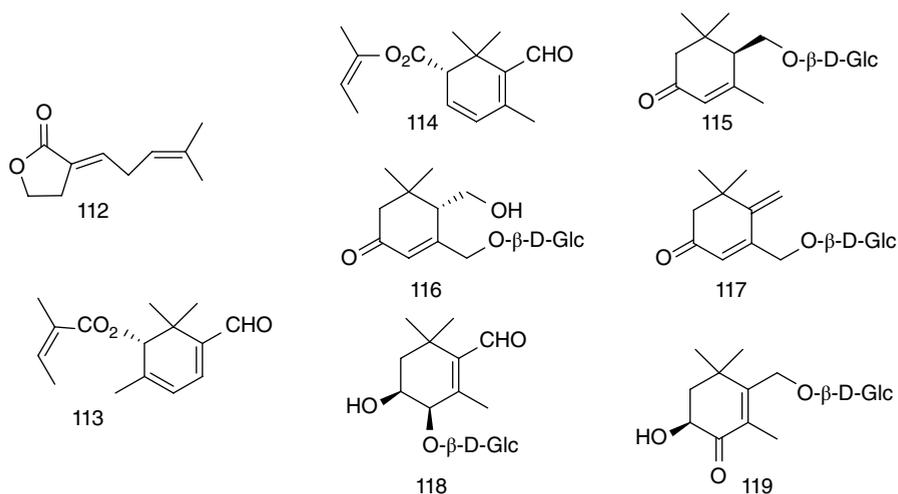


Figure 4.21 Some pyronene and cyclocitral derivatives.

(*R*)-(+)-limonene is the substrate, the maximal bioconversion of 45.8% has been observed using immobilised *P. digitatum* mycelia. In addition, the effects of cosolvent on this biotransformation have been investigated (Tan *et al.*, 1998a; Tan & Day, 1998).

In the other study, some dihydrocarveol (146) Mitsunobu reactions and its enantiomers have been reported. The photolytically induced radical fragmentation of β-hydroxyazide (147) causes the production of ketonitrile (149). The reaction between unsaturated

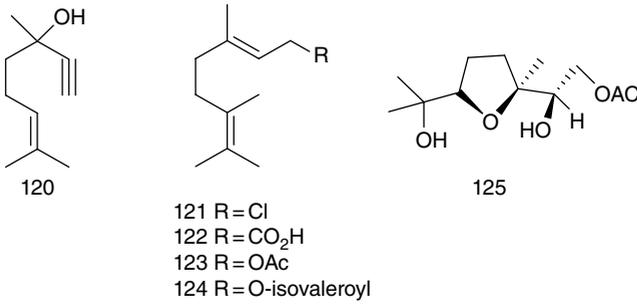


Figure 4.22 The structures from Pd–Al₂O₃ catalyzed hydrogenation of dehydrolinalool.

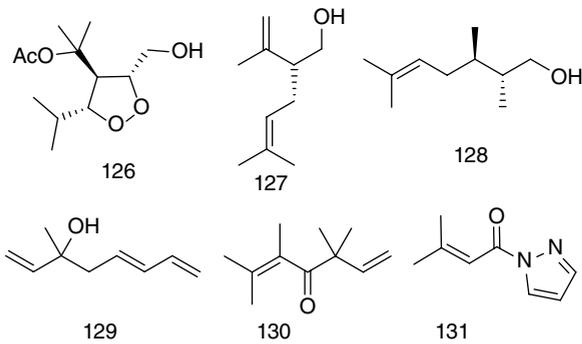


Figure 4.23 Chemical structure of some terpenes and terpenoids.

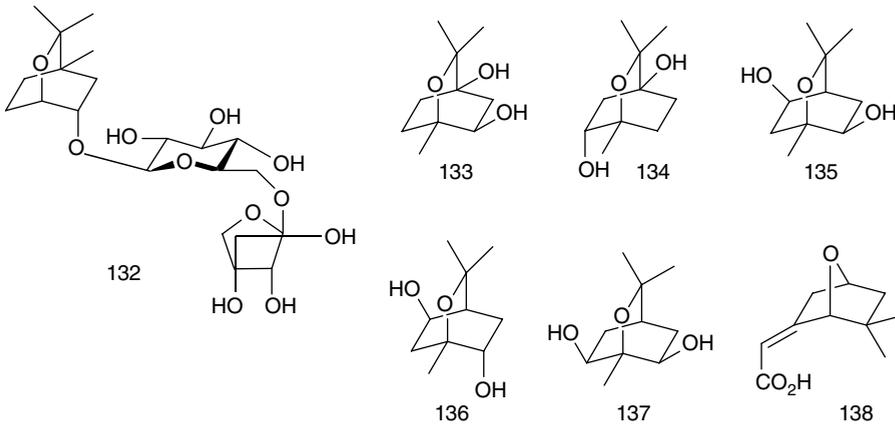


Figure 4.24 Some glycoside derivatives extracted from plants.

alcohol (150) and salicylaldehydes catalyzed by to askanite-bentonite clay to give (148) (Figure 4.26) (Shull *et al.*, 1997; Hernandez *et al.*, 1997; Volcho *et al.*, 1997).

According to Figure 4.27, the fungi *Glomerella cingulate*, *Rhizoctoniasolani* and *Aspergillusniger* can metabolise (–)-Isopinocampheo (151) and its (+)-enantiomer *ent*-(151). the (–)-alcohol (151) is converted into a mixture of the diols (152) and (153) and

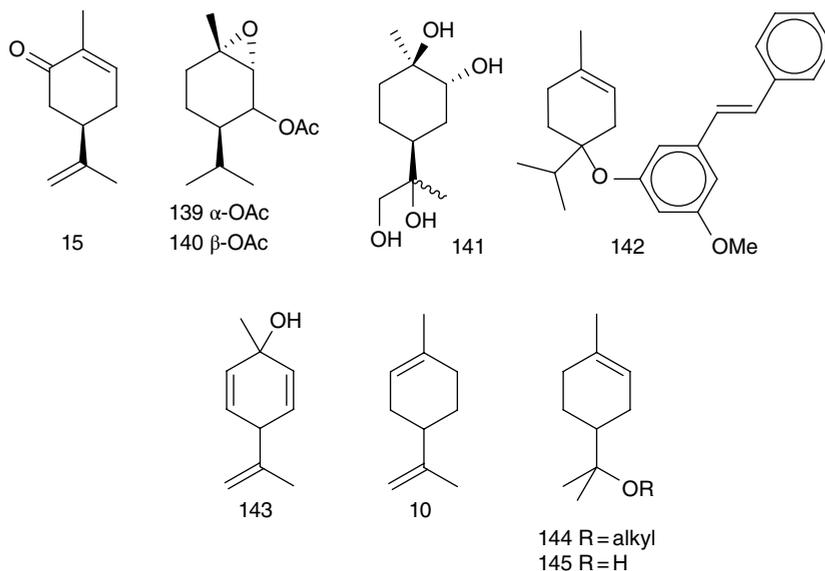


Figure 4.25 The structure of some obtained menthanes.

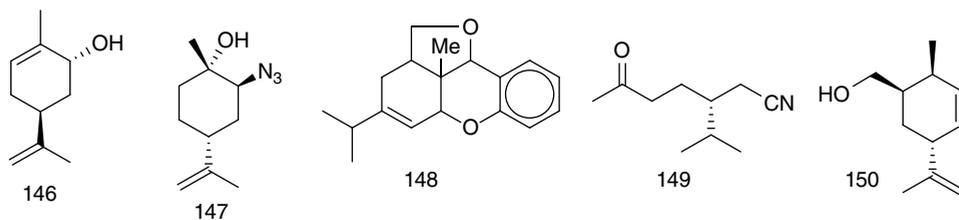


Figure 4.26 Some molecules observed via Mitsunobu reactions.

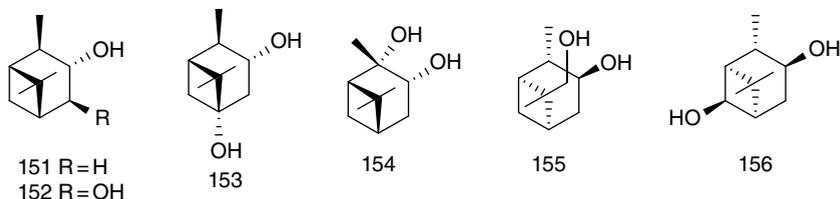


Figure 4.27 The chemical structures frommetabolisation of *Glomerella cingulate*, *Rhizoctoniasolani* and *Aspergillusniger*.

pinane-2,3-diol (154), when *G. cingulate* is applied, whereas (+)-(151) affords *ent*-(153) and the diols (156) and (155) (Miyazawa *et al.*, 1997).

In the optically active molecules, two enantiomers are commonly observed. These enantiomers are usually present in a variety of plants like (+)- α -pinene from *Pinuspalustris*, (-)- β -pinene from *Pinuspinaster* and from *Pinuscaribaea*, (+)-linalol from some camphor trees, and (-)-linalol from coriander (Figure 4.28). In some types the racemic form

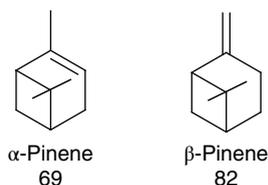


Figure 4.28 The structure of α - and β -Pinen.

is found; for example the structure (–) is more usual to rose and the isomer (+) is characteristic of *Eucalyptus citriodora*, and (\pm)-citronellol is widespread (Bakkali *et al.*, 2008). Enantiomers have varies organoleptic features. Herein, the absolute configuration of terpenes determines the application of which as flavour and fragrance (De Carvalho & Da Fonseca, 2006).

Distillation of turpentine oils proposes a pure practical group entitled α -pinene that takes part in many organic reactions and serves in the fragrance industries to improve the odour of industrial products. For example, pinane as an essential starting material in the industrial reaction is produced by the conversion of α -inene and has been used in the flavour and fragrance industries. α -Pinenes can also be isomerised to β -pinenes with high affinity to synthesis β -pinene (Kaiser, 1976). The ring opening hydration yields *cis*-terpin hydrate and terpineol. In the other process, the pyrolysis of α -pinene leads to a mixture of alloocimene and ocimene. With the respect to previous observations, it is less important as a precursor in industrial processes, for example, camphor, borneol, and terpineols. β -Pinene has as same reactions as α -pinene such as pyrolytic cleavage to yield myrcene. It uses as starting material for acyclic terpenes that are utilised in the industrial scales. Addition of formaldehyde results in the formation of nopol and nopyl acetates are served as fragrance components. Similar to α -pinenes, the distillations of turpentine oils will synthesis β -pinene in the large scales. Although most β -pinenes are applied in the production of myrcene, in the similar manner to α -pinenes, it has been used as fragrance compounds in household perfumeries (Surburg & Panten, 2006).

The production of enantiomerically pure flavours and fragrances and the stereo- and regioselective production of so-called ‘natural compounds’ can be achieved by biotransformation under mild reaction conditions. Bioconversion of terpenes is the second approach to synthesis commercially worthy pharmaceutical stereo isomers and chemical building-blocks (De Carvalho & Da Fonseca, 2006). The qualitative studies of the non-volatiles that were obtained from α -pinene reaction (157) with hydroxyl radicals have been done, and rate constants for these reactions with chlorine atoms and radical anions and also hydroxyl radicals, have been measured (Vinckier *et al.*, 1997; Kluge *et al.*, 1998). (–)- α -Pinene *ent*-(69) has been metabolised to a mixture with both of *trans*-verbenol (158) and verbenone (157) by the *Hormonemas* sp. UOFS Y-0067. The same microorganism can oxidise (–)- β -Pinene *ent*-(82) to produces pinocamphone (159) that can be further metabolised to 3-hydroxypinocamphone (160) (Figure 4.29) (Grayson, 2000).

There are some camphanes that including compounds (161–163) have been given from roots and rhizomes of *Glehnialittoralis* (Figure 4.30) (Kitajima *et al.*, 1998b). $\text{SiCl}_4/\text{K}_2\text{CO}_3$ or Ti(IV) oxodichloride are utilised in chlorinated mediums as worthy reagents for the conversion of alcohols like borneol (164) into the corresponding chlorides (Figure 4.29) (Ha & Chai, 1997; Ha & Kim, 1997). The reaction between SiCl_4 and

K_2CO_3 leads to trichloro siloxy carbonyl chloride (Cl_3SiO_2COCl), which can be used as a chlorinating agent, and reaction of extra $SiCl_4$ with K_2CO_3 compose triphosgene.

Conformationally rigid camphanes are used as reagents to progress asymmetric syntheses. It has been defined a practical approach for the stereo- and regioselective conversion of (-)-camphorquinone (165) into the diol (Ruzicka & Capato, 1925) as a useful chiral auxiliary. α -hydroxy ester (168) is generated from the glyoxalate (167) by reducing process using metal hydride reagents. It is observed that there is poor diastereoselectivity when sodium borohydride is used, but $LiAlH(^tBuO)_3$ at $0^\circ C$ affords (*R*)-configured yield, and *L*-selectride (lithium tri-*sec*-butylborohydride, 1.0 molar solution in tetrahydrofuran) at $-78^\circ C$ produces (*S*)-product. Both of which were in similar diastereomeric purity of $>96\%$ de (diastereomeric excess) (Figure 4.31) (Verdaguer *et al.*, 1998; Chen *et al.*, 1999).

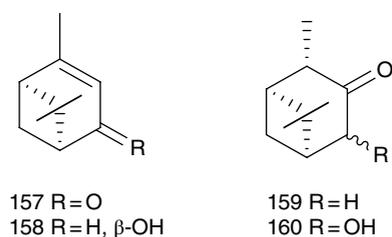


Figure 4.29 Some structures produced from α - and β -Pinen.

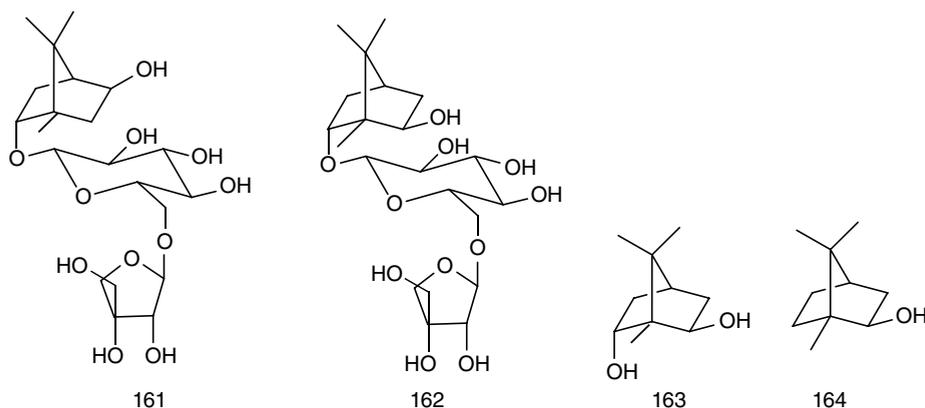


Figure 4.30 Some new camphanes from roots and rhizomes of *Glehnialittoralis*.

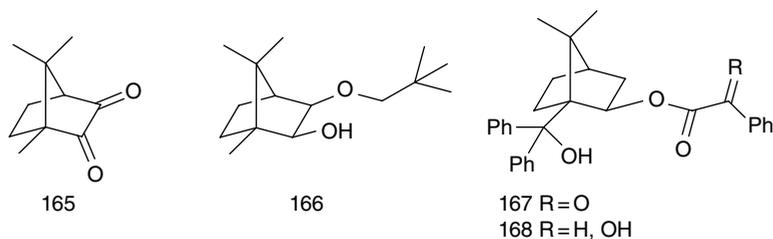


Figure 4.31 Rigid camphanes that have been applied to progress asymmetric syntheses.

It has also been investigated the synthesis of unsaturated tertiary thiols (169) and (170) and gas phase photochemistry and the solution of thiofenchone derivatives and different thiocamphor (171) have been reported (Figure 4.32) (Martinez *et al.*, 1997; Morrison *et al.*, 1998). 1,2-dithiine (173) were obtained by the conversion of bis-thiocamphor (172) *via* stereospecific rearrangements when iodine irradiates compound (172) (Figure 4.32) (Salama & Poirier, 1997).

The carane derivatives (174) and (175) possess acaracidal activity against the *Dermatophagoides farinae* and mites *Tyrophagusputrescentiae* more than *N,N*-diethyl-*m*-toluamide (DEET) (Figure 4.32) (Nomura *et al.*, 1998). A mixture of the three vinylic bromides (177–179) were presented by the solvolysis of dibromocarbene adduct (176) in methanol (Figure 4.32) (Brenna *et al.*, 1998). Some glycosides such as (180 and 181), have been isolated from *Foeniculumvulgare* (Figure 4.33) (Ishikawa *et al.*, 1998a).

3-Hydroxy-7,8-didehydro- β -ionol-9-*O*- β -D-glucopyranoside (182) were obtained from rose petals, and 4-Hydroxy- β -ionone (184) has been observed in *Chloranthusspicatus*. On the other work, some normegastigmane derivative (183) has also been elicited from *Wahlenbergiamarginata* (Figure 4.34) (Straubinger *et al.*, 1997; Tan *et al.*, 1998b; Huang & Yang, 1998).

Geranyldiphosphate can be converted into (-)-sabinene (185) by *Conocephalumconicum* (a European strain of the liverwort). There have been studied some biohydroxylation processes of (-)- α -thujone (186) in the literature (Figure 4.34) (Adam & Croteau, 1998, Ismaili-Alaoui *et al.*, 1997). The synthesis and biosynthesis of iridoid lactones have been reviewed (Figure 4.35) (Nangia *et al.*, 1997). In addition, the antirrhinolide (187), which found from *Antirrhinum majus*, has been synthesised. The imide linavuline (188) derivative has been isolated in *Linariavulgaris* (Figure 4.34) (Hua *et al.*, 1997).

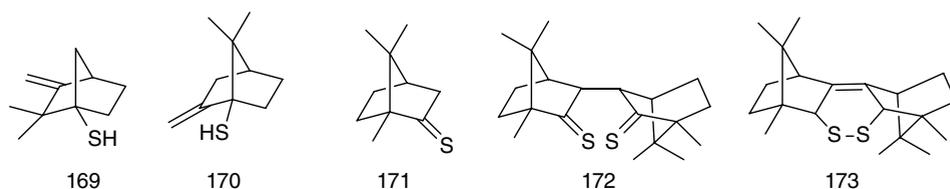


Figure 4.32 The structure of some synthesised unsaturated thiols.

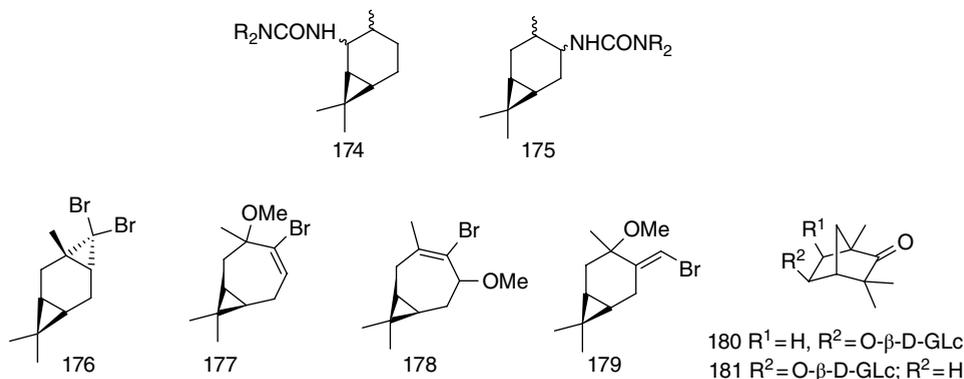


Figure 4.33 Chemical structures of some carane, vinylic bromides and glycoside derivatives.

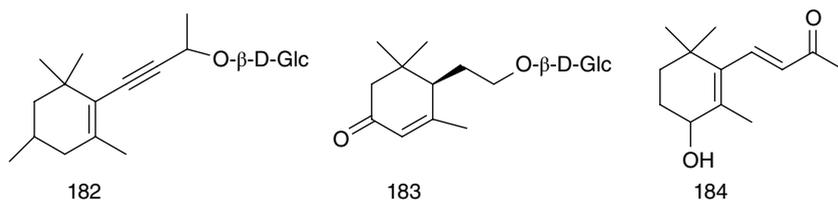


Figure 4.34 Some normegastigmane derivatives.

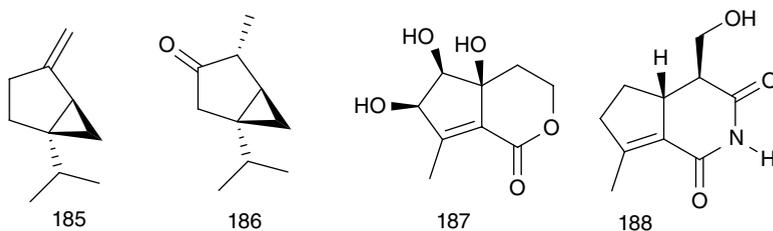


Figure 4.35 Chemical structures of (-)-sabinene, (-)-α-thujone, antirrhinolidide and imide linavuline.

4.3.2 Sesquiterpenes and Sesquiterpenoids

Enzymatic reactions are an important group of biosynthetic pathways to produce EOs (Eom & Hyun, 2016; Pazouki *et al.*, 2016). The production of monoterpenes is monitored by several terpene synthases (Picard *et al.*, 2017). Sesquiterpenes can also be synthesised from farnesyl diphosphate (C15, FPP) by enzyme sesquiterpene synthases (Chen *et al.*, 2016); but structurally, they are more diverse than monoterpene scaffolds. These enzymes have been recognised in plants, which are responsible for the biosynthesis of sesquiterpens (Fattahi *et al.*, 2016).

There are several sesquiterpene synthases including kunzeaol synthase (Pickel *et al.*, 2012), epi-cedrol synthase (Mercke *et al.*, 1999), *t*-cadinol synthase (Jullien *et al.*, 2014), and drimenol synthase (Kwon *et al.*, 2014) that have been investigated in the literature. They can convert FPP to the corresponding sesquiterpene such as kunzeaol, epicedrol, *t*-cadinol and drimenol (Gou *et al.*, 2016).

The features of sesquiterpenes are retrieved latest from wide spectrum of varies skeletal patterns in the chemistry of which. Farnesol as a natural sesquiterpene alcohol in EOs, has potential for alleviating oxidative stresses, massive inflammations and lung injuries. It can additionally amend motor coordination and neuromuscular strength while neurotoxicity induced by acrylamide. Farnesol compounds are adequate scaffolds for reprogramming prenyl metabolic origins. They can modify the homeostasis of interferes and plastids through MEP pathways. In addition, these skeletons behave as inhibitor and activator for the MEP and MVA pathways, respectively (Huchelmann *et al.*, 2016; Santhanasabapathy *et al.*, 2015).

The most substantial intermediate in sesquiterpenes biosynthesis is farnesol. This fact has created an interesting motivation to scientists to find sufficient procedures for the synthesis of acyclic sesquiterpenes (Santhanasabapathy & Sudhandiran, 2015; Ku & Lin, 2016). Scientists have reported an interesting method for synthesising of transnerolidol (193) from cyclopropyl methyl ketone (189). The reaction between this ketone

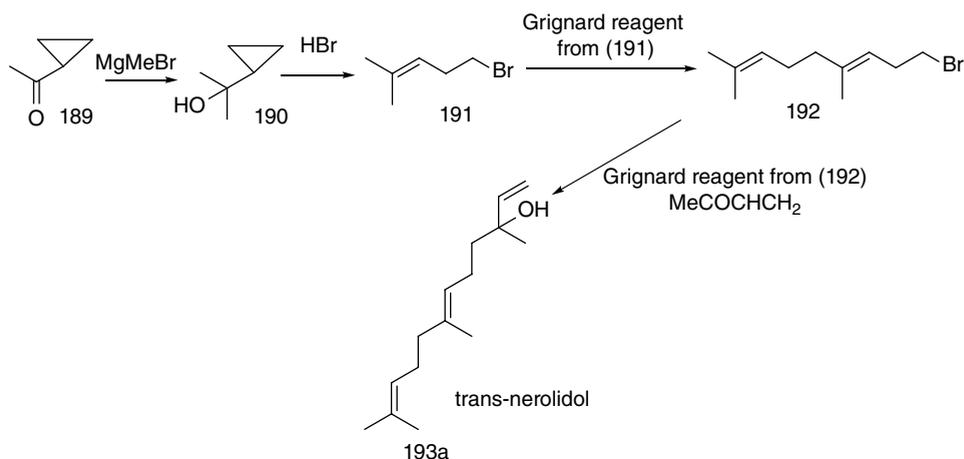


Figure 4.36 Synthesis of polyisoprenoid.

with methyl magnesium bromide makes the alcohol (190) that was converted into the bromide (191) by hydrogen bromide. This sequence can be repeated to give bromide (192) which makes pure trans-nerolidol (193) with methyl vinyl ketone. Thus, it is possible to synthesise polyisoprenoids in a facile sequence of reactions (Figure 4.36) (Mellor & Munavalli, 1964).

Farnesol hoarded at concentrations which are toxic for other cells. It can function as an efficient compound to compete for available FPP pool applied for the biosynthesis of (*E*)-nerolidol sesquiterpene (Huchelmann *et al.*, 2016). Treatment of nerolidol (193a, 193b) with acid prepared a crude of acyclic molecules by the cyclisation of monocyclic bisabolene (194) and bisabolol (195). This traditional synthesis has many difficulties separating the isomeric products. In the synthesis of structures containing the bisabolane units, it must be noticed that all the coincidence of isomers physical constants had been formed naturally and those of the synthetic products is fortuitous. However, almost over of difficulties are resolved by chromatographic techniques that have been developed to facilitate the separation of isomeric mixtures. All syntheses through both aromatic and alicyclic intermediates are possible. Thus, the Birch type reduction of intermediate (197) (6-*p*-methoxyphenyl-2-methylhepta-2,5-diene), gives zingiberene (196) which is a configurational isomer of bisabolene (194) (Figure 4.37) (Mellor & Munavalli, 1964).

The synthesis of cadalene (198) as a completely aromatic dehydrogenation product of cadinanes, confirmed the structure of this cadinane skeleton itself. The further dehydrogenation gave calamenene (199) has been obtained from γ -(5-isopropyl-2-methylphenyl)- α -methylbutyric acid with ring closure followed by ketone (200) transformation. Diketone (201) is an essential intermediate for subtle synthesis in cadinanes. The essential problem at present is the stereochemistry monitoring of the configuration and of the ring junction of substituents, absent in the dehydrogenation products production. This synthetically racemate intermediate (201) synthesised from 4-isopropyl-6-methoxy-1-tetralone (202), or as the (-)-isomer, prepared from (-)-cryptone (203). This observation provides the authority of extension to other cadinane derivatives. It has been already converted into (\pm)-cadinene hydrochloride (204) (Figure 4.38) (Mellor & Munavalli, 1964).

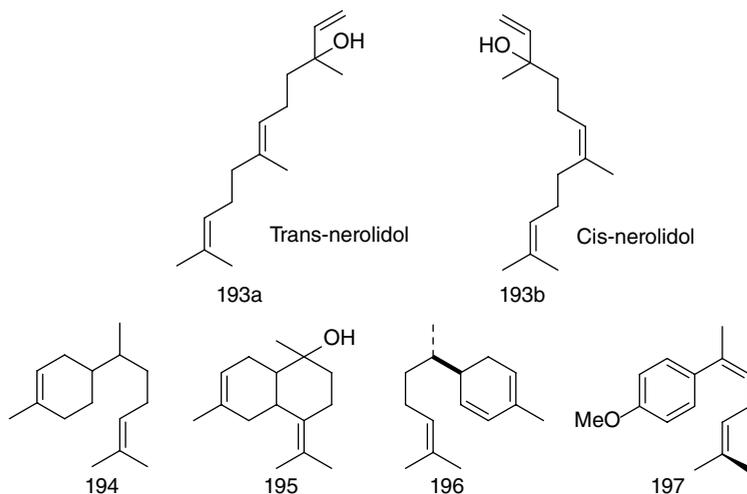


Figure 4.37 Chemical structures of nerolidol, bisabolene, bisabolol, zingiberene and 6-p-methoxyphenyl-2-methylhepta-2,5-diene.

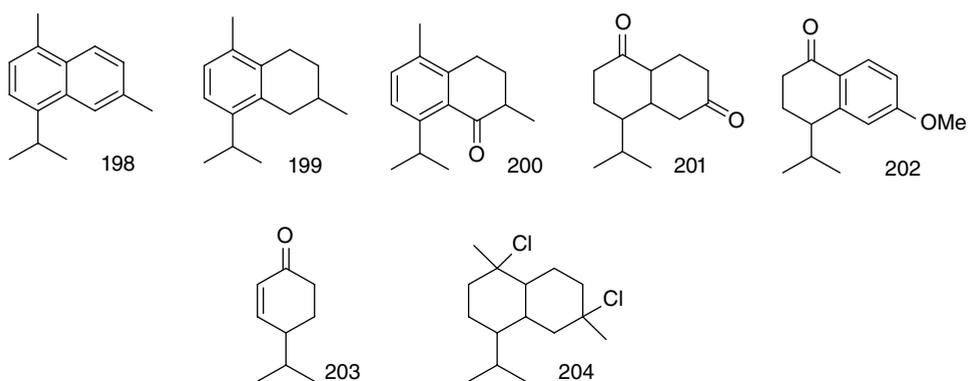


Figure 4.38 Chemical structures of cadalene, calamenene, ketone and diketone intermediate in cadinanes synthesis, 4-isopropyl-6-methoxy-1-tetralone, (-)-cryptone and (±)-cadinene hydrochloride.

The key step in successful clovene (205) synthesis is the utility of internal condensations. It is further illustrated by the synthesis of acorone (206) and cedrol (214) (Figures 4.39 and 4.40). Norcedrene dicarboxylic acid (210; R=H) was applied as an auxiliary in the cedrolsynthesis (Stork *et al.*, 1961). Thus, the synthesis has two sensitive stages including the skeleton establishment of norcedrene dicarboxylic acid and the acid alteration into the tricyclic cedrol component. The cyclo pentenone diester (207) was utilised as the elaboration and precursor by standard approaches made the diester (208). The Claisen condensation caused the bicyclic structure, giving molecule (209) followed by conversion into norcedrene dicarboxylic acid to quench the first section of the process. A base cyclise the keto-ester (211) synthesised from mentioned acid to the tricyclic β -diketone (212), to affect the second essential cyclisation reaction. Both reduction of enol ether crude from diketone (212) and direct reduction with LiAlH_4

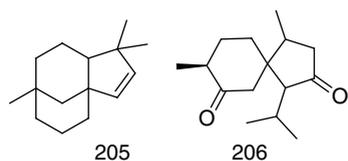


Figure 4.39 Chemical structures of cloven and acorone.

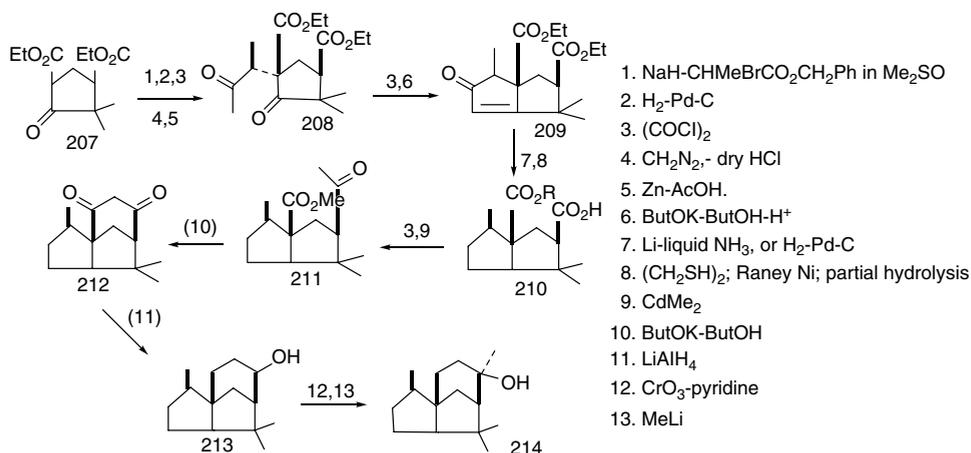


Figure 4.40 Synthesis of cedrol.

gave the alcohol (213) was altered into cedrol (214) in two more stages. This synthesis process is investigated and characterised by the high level of stereo specificity in the varied condensations and alkylations (Figure 4.40) (Mellor & Munavalli, 1964).

As explained previously, these types of EOs contain 15-carbon skeleton. Their volatilities are lower than monoterpenoids, therefore, their boiling points are higher than which are. Hence, fewer percentages of sesquiterpenoids assist to the odour of EOs but those do usually have the aroma of less and significantly are contributed as endnotes. They have been also used as promising fixatives for more volatile ingredients. While geraniol (67) is utilised as precursor for the synthesis of monoterpenoids, farnesol (215) is commonly applied for sesquiterpenoids production (Figure 4.40). As illustrated in Figure 4.15, the treatment of geranyl pyrophosphate (74) with isopentenyl pyrophosphate (72) in nature gives its pyrophosphate and farnesol will be generated by following hydrolysis of that. The primary carbon-oxygen bond heterolysis of phosphate produce a borning farnesyl carbocation (216) (Figure 4.41) and this causes the production of different sesquiterpenoids as same as the geranyl carbocation leads to monoterpenoids. The variety of cyclic molecules derived from farnesyl pyrophosphate and a scope for more structural variations resulting from degradation, oxidations, and rearrangements, are more than ones from geranyl pyrophosphate due to farnesyl pyrophosphate has three double bonds in the skeleton. In this process, double bond geometry in the position 2 of farnesol is essential for recognising the utilised procedure for the reactions of subsequent cyclisation (Baser & Buchbauer, 2015).

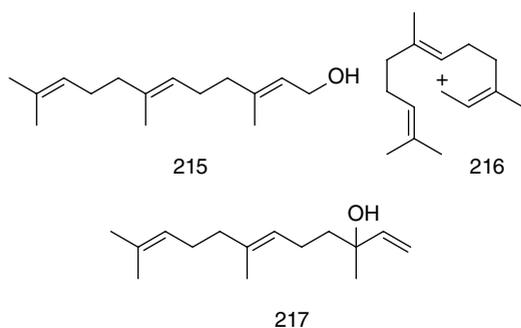


Figure 4.41 Three precursors for sesquiterpenoids.

Figure 4.42 shows schematic biosynthetic approaches based on (*Z,E*)-farnesyl pyrophosphate. Direct hydrolysis produces farnesol (215) and nerolidol (217) while, the capture of carbocation (216) by double bond located at position 6 leads to cyclic structures of the bisabolane (218) and so, bisabolol (219) is synthesised by quenching of (218) with water. In the other pathway, the isomeric carbocation (220) will be generated by hydrogen shifting in (218) and retains the bisabolane skeleton. Further rearrangements and cyclisations take the compound via different skeletons such as those of the cedrane (222) and acorane (221) families, to the khusane species, exhibited by khusimol (223). A great spectrum of materials can specifically be obtained along this path. For example, cedrol (224) is being synthesised by treatment of cation (222) with water. Other double bonds in the structure can be also prepared by cyclisation of the bisabolyl carbocation (218) leading to the campherenane skeleton (225), α -santalol (226), and β -santalol (227) or via the chamigrane (229) and cuparane (228) skeletons, to molecules like thujopsene (230). The carbocation function in (216) can add to double bonds at far end of the scaffold chain to prepare the *cis*-humulane (231). The sekinds give caryophyllene (232) due to they can cyclise back to double bond at carbon 2 before releasing a proton. Other alternatives are for a series of rearrangements, cyclisations, and hydrogen shifts to create it via the longibornane (234) and himachalane (233) skeletons to longifolene (235) (Baser & Buchbauer, 2015).

Figure 4.43 shows some other pathways for sesquiterpenoids biosynthesis using (*E,E*)-farnesyl pyrophosphate. Although, cation (236) cyclisation to C-11, followed by proton loss leads to α - or *trans*-humulene (237), cyclisation to alternative end of identical double bond produces carbocation (238) with the germacrane structure, which is being served as an intermediate in various odourous sesquiterpenes biosynthesis like α -vetivone (241) and nootkatone (239). β -Vetivone (241) is composed by an approach that also generates a wide variety of alcohols such as molecules (242), (243), and an ether (244), which has the eudesman structure. Germacrane carbocation rearrangement (238) gives a carbocation bearing the guaiane skeleton (245) and this has been recognised as an intermediate in guaiolsynthesis (246). Carbocation (245) has been also used as an intermediate in the biosynthesis of patchouli alcohol (249) and of α - and β -patchoulane skeletons (247 and 248, respectively) (Baser & Buchbauer, 2015).

It is observable both ends of β -carotene chain (20) have been cyclised to produce cyclohexane rings. Several fragments are found in EOs and the two main groups of which are damascones and ionones. These fragments can be generated by central part

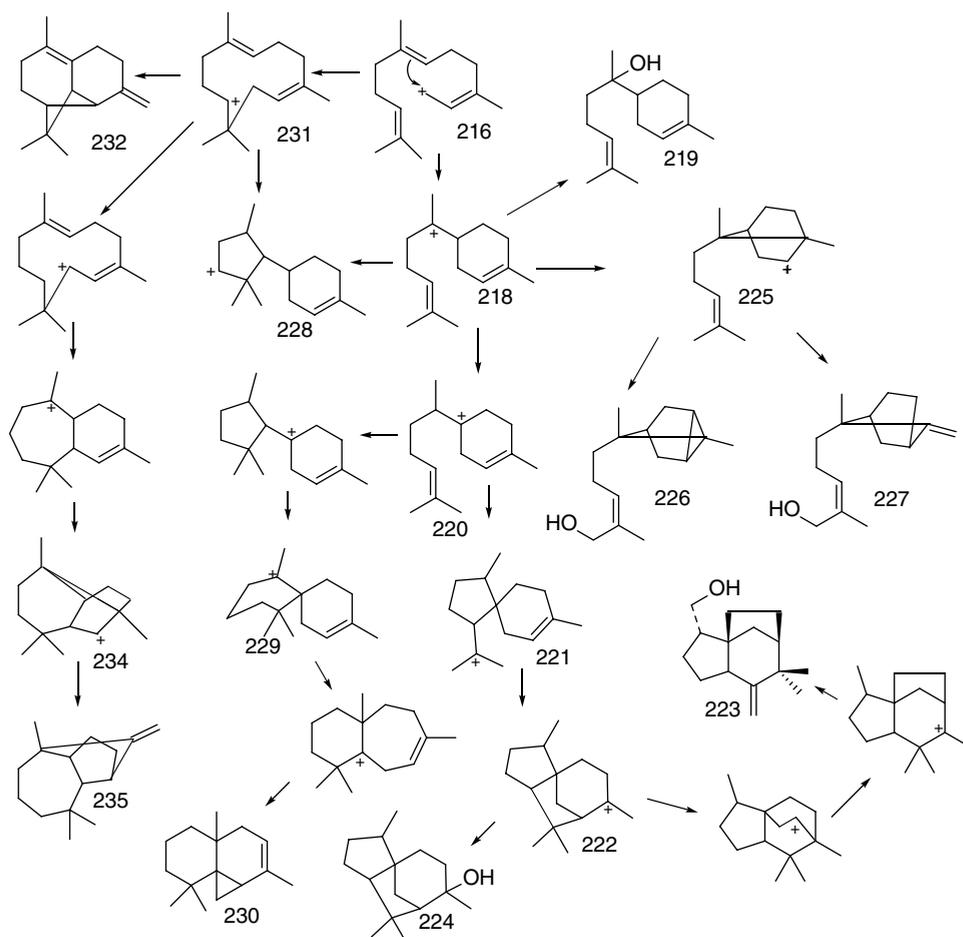


Figure 4.42 Several biosynthetic approaches from *(Z,E)*-farnesol.

degradation of chain. Although the carbon skeleton of ionones is as same as the damascones, the oxygenation in damascones is carried out at the chain adjacent to ring and in the ionones, oxygenation site is located at three carbon atoms far from the intended ring (Figure 4.44) (Sell, 2003). A great spectrum of leaves, fruits, and flowers presents ionones, which are the most important ingredients of perfumes (Sell, 2007).

Compounds α - (250) and β -ionones (251) form around 57% of the volatile ingredients of violet flowers and both of them remarkably occur in the nature. The damascones are also obtained from a wide diversity of flora, which occur usually at very low rates. However, their very intense odours clarifies that they have striking portion of oils containing them. β -damascenone (252) was obtained at a level of 0.05% in Damask rose oil. Compound (252) and the α - (253) and β -isomers (254) have since been identified in various extracts and EOs. The side chains of cyclocitral (257) and safranal (255) are degraded from more carbon sites that attached to cyclohexane rings. Safranal has around 70% of saffron volatile ingredients that makes a considerable portion to their odour. Other degradation contingences of volatile carotenoid components occur in EOs and are responsible for their odours including

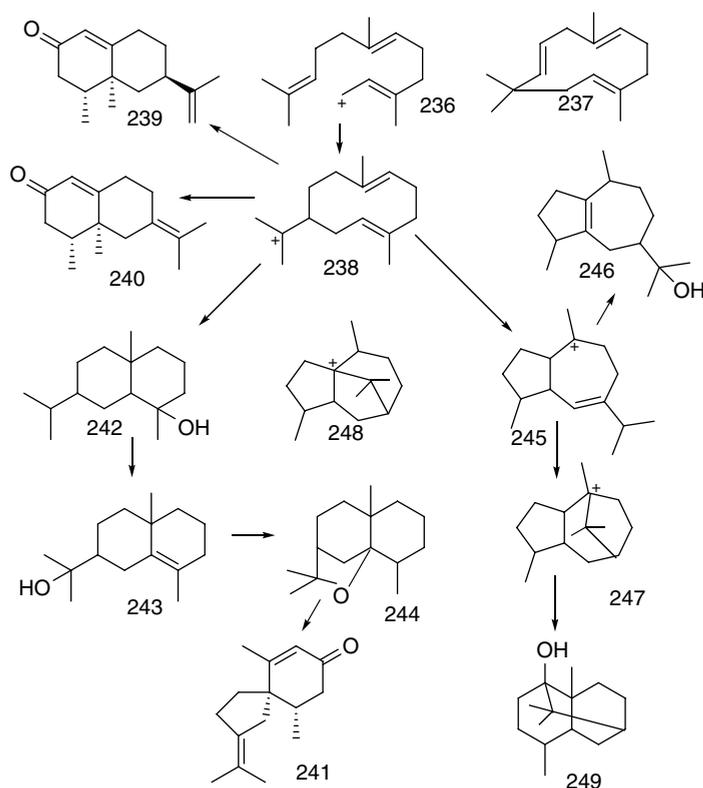


Figure 4.43 Some possibilities that biosynthetically utilised (*E,E*)-farnesol.

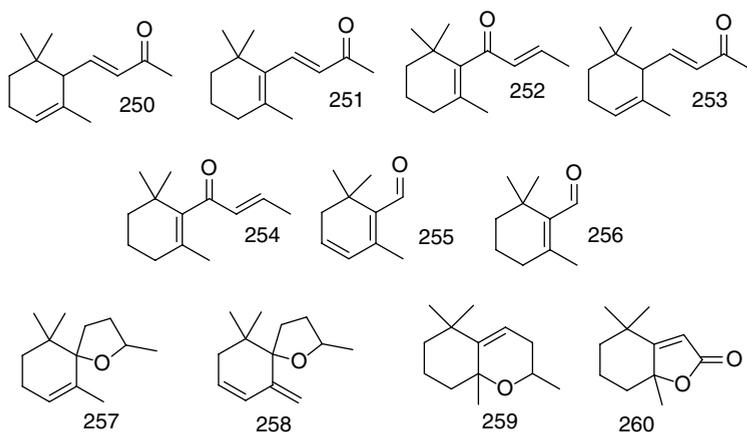


Figure 4.44 Carotenoid degradation products.

vitispiranes (258), theaspiranes (257), dihydroactindiolide (260) and edulans (259) (Baser & Buchbauer, 2015).

Many esters, aldehydes and alcohols containing a 2-methylbutane scaffolds, happen as minor ingredients in EOs. In biosynthesis fields, the most popular oxidation pattern is about prenyl, which is, 3-methylbut-2-ene-1-ol. For an practical example, the acetate

form of mentioned alcohol happens in the both ylangylang and several other oils. However, all positions can do oxidation process. Prenyl acetate as a one of esters generates fruity top notes to oils comprising these acetates and corresponding thioesters take part to the odour of galbanum (Baser & Buchbauer, 2015).

4.3.3 Aromatic Compounds

The second largest family of plant volatiles (PVs) includes components involving an aromatic ring. Although, it has been shown that not all reactions lead to basic skeletons synthesis, most of them can be derived from intermediates in method. These reactions lead to phenylalanine followed by primary and secondary nonvolatile compositions from shikimate (Dudareva *et al.*, 2004). The starting material for lignin is eugenol (clove essence), which is the reduced exemplar of coniferyl alcohol (Ganget, 2001). Phenylacetaldehyde is found in tomato fruits and obtained from phenylalanine by decarboxylation reaction and oxidative elimination of amino group (Tadmor *et al.*, 2002; Hayashiet, 2004). Aromatic building blocks like benzaldehyde and benzoic acid are generated by shortening of the three-carbon side chain of hydroxycinnamates (derived from phenylalanine) to one carbon (Borejsza-Wysooki & Hrazdina, 1996). Many other PVs are efficiently prepared by type III polyketide synthases (PKSs), which consume malonyl- and cinnamoyl- coenzyme As (CoAs) as precursor (Chen & Ho, 1988).

The formation of phenolic rings through condensation of polyketidesis exhibited in Figure 4.45. Intramolecular aldol condensation of tri-ketooctanoic acid followed by enolisation produces orsellinic acid (261). Polyketide type phenols are distinguished from shikimate phenolic systems according to this fact that the former retains the evidence of oxygenation process on various carbon atoms. Ethyl everninate (263) and methyl 3-methylorsellinate (262) are the most notable in odour concept and are usually also found in the acceptable scale (Baser & Buchbauer, 2015).

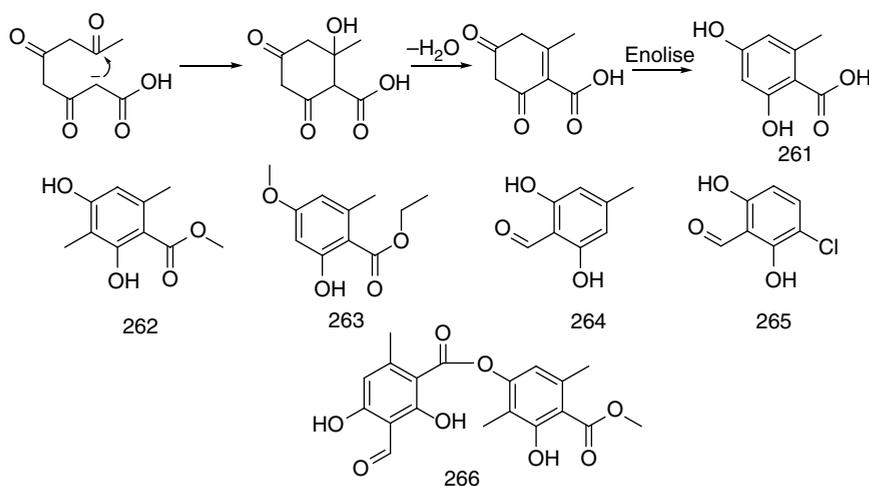


Figure 4.45 Polyketide biosynthesis and oakmoss components.

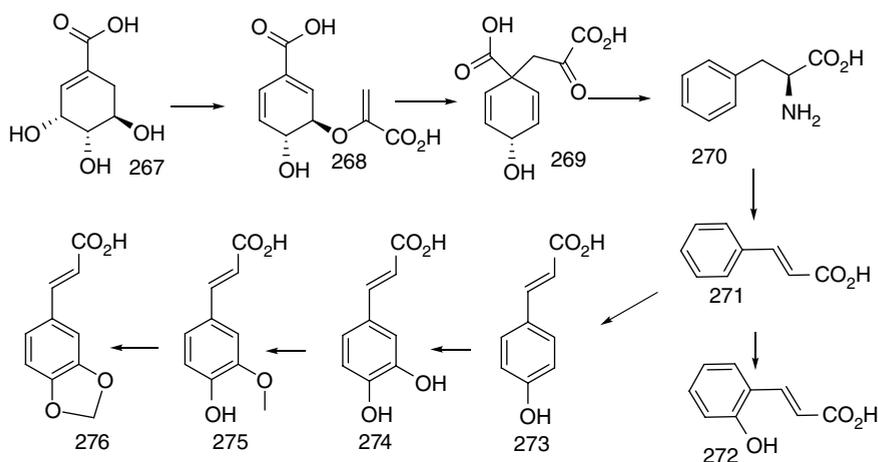


Figure 4.46 Key intermediates from shikimic acid.

Shikimic acid (267) as the precursor for the biosynthesis of key intermediates is illustrated in Figure 4.46. The importance of these intermediates is in generating substances volatile enough to be essential oil constituents. chorismic acid (268) is generated by deletion of one of the ring alcohols and reacted with phosphoenolpyruvate (28). Then, an oxy-Cope reaction converts chorismic acid (268) to prephenic acid (269). Phenylpropionic acid skeleton is result of elimination and decarboxylation of the ring alcohol. The essential amino acid phenylalanine (270) come from amination and reduction of the ketone function whereas reduction and elimination give cinnamic acid (271). Subsequently, ring hydroxylation leads to the isomeric *o*- and *p*-coumaric acids (272) and (273). Caffeic acid (274) is result of further hydroxylation and methylation of which produces ferulic acid (275). Methyl ether oxidation and subsequent cyclisation leads to methylenecaffeic acid (276). Depending on the plant genetic structure, it is often feasible to reach to intended product through varies sequences of identical procedures in shikimate biosynthesis (Baser & Buchbauer, 2015).

4.4 Effective Factors on the Composition of Essential Oils

Fermentation, enfleurage, expression or extraction are methods for obtaining essential oils, but steam distillation method is often applied for the commercial production of different EOs.

It is well known that steam distillation conditions lead to chemical change of monoterpenes and citrals (a pair, or a mixture of terpenoids with the molecular formula $C_{10}H_{16}O$) (Chen & Ho, 1988). Even formal solvent extraction is likely to involve loss of the more volatile details during emitting of the solvent by distillation (Bartley & Jacobs, 2000).

Extraction in the liquid carbon dioxide condition under high pressure and low temperature produces organoleptic profiles but is so expensive (Moyler, 1998).

The EOs composition can differ between geographical sources and between harvesting seasons from a particular species of plant (McGimpsey *et al.*, 1994; Arras & Grella,

1992; Marotti *et al.*, 1994; Cosentino *et al.*, 1999; Marino *et al.*, 1999; Juliano *et al.*, 2000; Faleiro *et al.*, 2002).

In species of *Thymus* and *Origanum*, 1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene (γ -terpinene) and 1-methyl-4-(1-methylethyl)-benzene (p-Cymene) are the starting materials of 5-methyl-2-(1-methylethyl) phenol (thymol) and 2-methyl-5-(1-methylethyl)-phenol (carvacrol) (Cosentino *et al.*, 1999; Jerkovic *et al.*, 2001; Ultee *et al.*, 2002).

In Greek oregano plants, all these compounds have been found to be somehow equal in samples obtained from various geographical regions (Kokkini *et al.*, 1997) and also, to be remain very stable in plants harvested pending various seasons (Jerkovic *et al.*, 2001; Marino *et al.*, 1999). This shows clearly that the four structures are functionally and biologically supports this theory that p-cymene formes thymol via g-terpinene in *T. vulgaris* (Kokkini *et al.*, 1997). In general, EOs obtained from herbs during or after flowering have the strongest antimicrobial activities (McGimpsey *et al.*, 1994; Marino *et al.*, 1999).

It is observed that varies parts of the same plant will give different composition of EOs widely. For example, a special EO extracted from coriander (*Coriandrum sativum* L.) seeds has entirely different composition to which of cilantro that is extracted from immature leaves (Delaquis *et al.*, 2002).

EOs are volatile and sensitive to high temperatures required for stem and hydrodistillation, which usually used for EOs extraction. Therefore, these methods may lead to chemical alterations in all compositionsof EOs. The solution for this problem is extraction procedure using supercritical fluids, particularly carbon dioxide, as described by (Fornari *et al.*, 2012).

According to previous investigations, the main essential oils have revealed in the leaf of *E. dysenterica* species were sesquiterpenes (Costa *et al.*, 2000; Duarte *et al.*, 2008b).

Seasonal effects on oil chemo variations have been studied in cultivated individuals originated from seeds had been obtained from two sites (Duarte *et al.*, 2008b). Additionally, terpene dynamics variations during the ripening of fruit have shown that monoterpenes concentrations were high up to semi-ripe stage, and afterwards, stepped down. Subsequently in the ripening process, sesquiterpenes were intensively synthesised, whereas ester occurrence was negligible (Duarte *et al.*, 2008a). Despite of the striking potential and growing market for EOs, many of them that can be obtained from fruit and leaf are unknown to different industries such as cosmetics. Moreover, environmental and genetic effects on the chemical components diversity of their cultivated samples and different wild populations have not yet been observed (Duarte *et al.*, 2010).

For investigation on the effect of drying process on the chemical composition of EOs both dried and fresh ginger cases have been examined and the major influences of drying method are an increase in terpene hydrocarbons, a reduction in gingerol content, and monoterpene alcohols conversion to corresponding acetates (Bartley & Jacobs, 2000).

In Australia, and particularly in Queensland, most ginger is marketed as whole fresh rhizomes. However, a significant proportion of the crop is exported in a dried form, and the United Kingdom, one of the world's principal consumers of this spice, imports about 4000 t of the dried material annually. In some countries, drying is carried out in the sun (Lawrence, 1984b) but in Queensland, conventional two-stage drum driers are used. It has been proven that the drying processes cause the favour changes to the product, but the chemical identity of these changes is not obvious exactly (Bartley & Jacobs, 2000).

Drying process as an effective factor on the changes was examined and reported a decrease in monoterpenes and citrals relative to sesquiterpenes, presumably largely due to the differences in volatility of these classes of compounds (MacLeod & Pieris, 1984; McGraw *et al.*, 1984).

The chemically variations influenced by season are significantly clear in berry oils, in contrast to foliage oils. From spring to autumn the yield of oils in berries enhanced by 162%. During the summer, α -pinene amounts diminished in the foliage oil. Seasonal variations in the foliage oils are overlay much less than in the berry oils. Constituent amounts alterations were revealed with a comparison of oils from fresh versus dried berries and foliage. after drying, the amount of α -Pinene will decrease, whereas other constituent amounts in berry and foliage oils will be increased (Shanjanian *et al.*, 2010).

Obviously, it was asserted that plant drying demonstrates the changes of flavour to the oil (Bartley & Jacobs, 2000) and there are considerable oils loses during the process of drying. Thus, the substantial issue is the finding of differences in EOs from fresh and dried foliage and berries of different species containing EOs to recognise oil potentials for commercial purposes (Shanjanian *et al.*, 2010).

The influence of both genetic and non-genetic variables involving edaphic and climatic conditions on the quality and quantity of volatile oils is inevitable (Powell & Adams, 1973; Fluck, 1963).

Phytochemistry of the plant may be influenced by the season and even the hours number of sunlight due to this fact that some components may be gathered at a specific duration to respond to ecological changes (Koenen, 2001; Burbott & Loomis, 1967). For example, monoterpenes in peppermint are employed for energy metabolism while proper stored substrates become discharged within the secretory cells (Shanjanian *et al.*, 2010).

The oil cases from autumn planted crops had a remarkably lower terpinen-4-ol and higher concentrations of menthol than those from spring planted crops (Marotti *et al.*, 1994).

Genotype and agronomic conditions, such as plant age, harvesting time, soil fertility and crop density greatly influence on the quantitative composition of the essential oils of many aromatic plants (Cosentino *et al.*, 1999).

Two factors influence the oil composition quantitatively are planting time and mineral fertilisation. In comparison, the oils obtained from spring planted crops exhibited a remarkably the higher content of menthol ($P=0.01$) and lower terpinen-4-ol concentrations ($P=0.05$) with those from autumn planted crops. The menthol content of the essential oil enhances in mineral fertilisation compared with that of the untreated plants, may be due to lower rates of nitrogen and phosphorus. Oils from the treated crop exhibited in most cases also a higher percentage of pulegone and lower p-caryophyllene and menthone contents (Marotti *et al.*, 1994).

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5

Basic Structure, Nomenclature, Classification and Properties of Organic Compounds of Essential Oil

Iuliana Vintilă

Food Science, Food Engineering and Applied Biotechnology Department, University 'Dunărea de Jos' Galați, România

5.1 Introduction

The essential oils, volatile oils or ethereal oils are highly volatile complex of terpenes (monoterpenes, sesquiterpenes) and alcohols, esters, aldehydes, ketones, lactones, coumarins and ethers (Figure 5.1 and Figure 5.2) extracted from the odouriferous (aromatic) sources as a viscously liquid with a specific aromatic profile (Figure 5.3 and Figure 5.4) of characteristic volatile compounds and natural preservative properties in their own botanical structure.

Essential oils are plant metabolites synthesised by herb and spices, accumulated in cytoplasm as single or aggregated droplets of volatile and aromatic organic compounds or mixtures of fragrant and odourless natural substances.

The essential oils are highly volatile, oxygen, light- and heat-sensitive natural mixtures, well-preserved by encapsulation. The essential oil bioactivity were raised by microencapsulation for antibacterial activity of carvacrol against *Bacillus cereus* in milk, rosemary essential oil compounds against *Listeria monocytogenes* or clove oil–inhibiting action on *Salmonella enteritidis* (Burt, 2004; Djilani, 2012; Dohi *et al.*, 2009; Ghayenpour *et al.*, 2014; Hong, 2004; Jayant *et al.*, 2014; Khoudja *et al.*, 2014; Lin *et al.*, 2012; Sultana *et al.*, 2007; Zhang *et al.*, 2016).

Essential oils based on spices or herbs sources as oregano, mint, salvia or clove could penetrate through bio-membranes (Koul *et al.*, 2008; Lang *et al.*, 2012; Nerio *et al.*, 2010; Pitarakili, 2002, 2003; Steuer *et al.*, 2001; Tepe *et al.*, 2005; Yadava *et al.*, 1998), modify the permeability or the integrity of the cytoplasm or other cell structures (mitochondria, nuclear DNA) which explain the antimicrobial effect of essential oils compounds.

The essential oils are colourless to yellowish hydro alcoholic natural extracts with a specific mass of 0.84–1.18 kg m⁻², slightly water-soluble but highly oil-soluble mixtures.

The essential oils classification were realised according their application:

- essential oils used in perfumery, soap and cosmetic industry;
- essential oils used in food and beverages industry as flavourings and preservation additives;

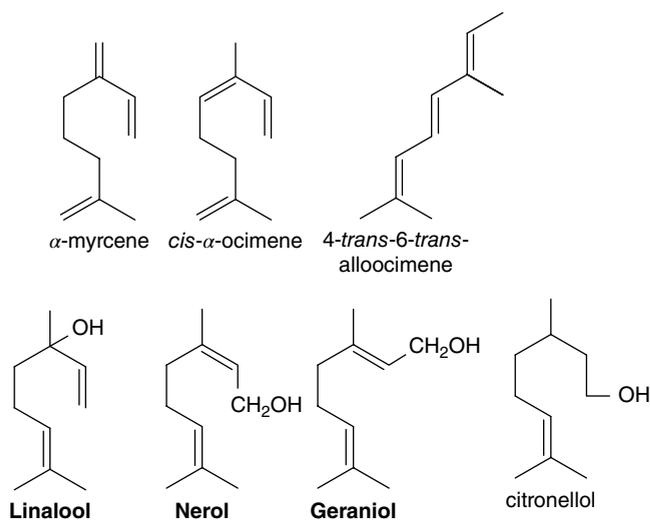


Figure 5.1 Monoterpenes from essential oils.

- essential oils used in agro-industrial proposes as anti-fungal, repellent, insecticidal lure, anti-feedant;
- essential oils used in medical (chemotherapeutical or biological active agents: analgesic, anti-anxiety, anti-inflammatory agent) and alternative medico-therapeutically techniques (massage, sauna, air freshening, vapour bathing, compresses, inhalations) in holistic medicine approach (homeopathy, hydrotherapy, massage therapy, *etc.*).

Also, a classification from the geographical origin and botanical sources is commonly used in case of essential oils.

ISO 4720, last revised in 2009, present the relevant plants as natural sources for obtaining specific essential oils.

The enantiomeric composition of essential oils compounds could be analysed by the following analytical methods: GC-MS, chromatographic methods of isolation and NMR chemical structure identification.

Generally, plant essential oils are mixtures of monoterpenes (1,8-cineole, α -pinene, camphor, camphene), sesquiterpenes and phenyl propanoids, polyphenols, alcohols (carvacrol, thymol, eugenol, falcarinol) with a molecular weight more than 500 Da (Adams, 2007; Liu *et al.*, 2006; Lopez *et al.*, 2007; Marceta *et al.*, 2012; Mazoochi *et al.*, 2012; Mohamede, 2014; Rajendran *et al.*, 2014).

In low percentage, linalool and linalyl acetate are the bioactive key compound in *Lavandula* essential oil and thymol in *Thymus zygis*. In high content, camphor and eucalyptol are the active compounds in *Rosmarinus officinalis* and in very high concentration the fenchone is the key bioactive substance in *Foeniculum vulgare* (Baser & Buchbauer, 2010; Bilia *et al.*, 2014; Gautam *et al.*, 2014; Lesgards *et al.*, 2014; Raut & Karuppayil, 2014; Seow *et al.*, 2014; Tongnuanchan & Benjakul, 2014).

The oily aromatic natural extracts represent the concentrated aromatic essence from a natural source usually an aromatic plant, condiments, fruits, seeds, flowers, stems, buds, roots, leaves or woods.

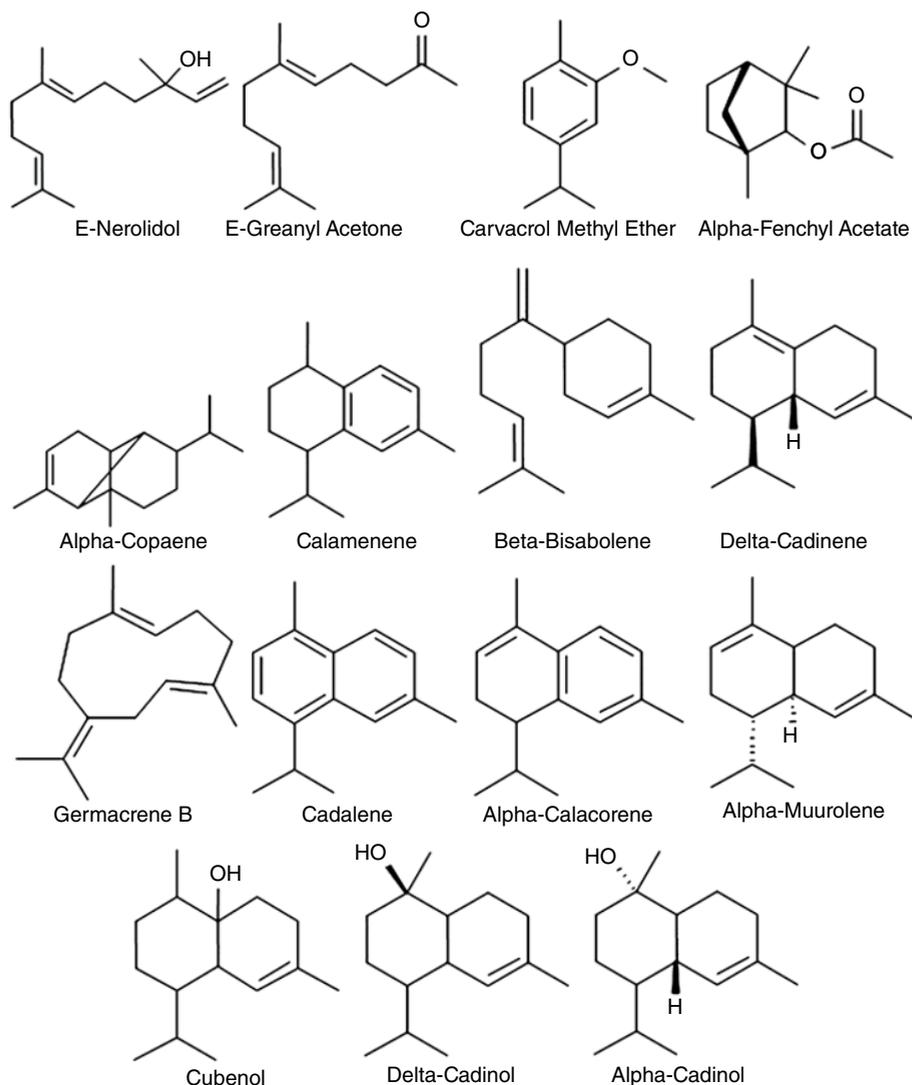


Figure 5.2 Sesquiterpenes from essential oils.

Essential oils are complex chemical structure because hundred of aroma compounds could be included in one plant extract as “quintessential oil” from a particular source: orange, eucalyptus, spearmint, lemon, chedarwood and menthe.

The basic structure and extraction yields are specific, depending on source and extraction method (Table 5.1 and Table 5.2).

The principal methods of essential oil production are as follows:

- 1) cold pressing, expression;
- 2) distillation: steam distillation, water and steam distillation, fractional distillation;
- 3) extraction: maceration, solvent extraction, supercritical extraction.

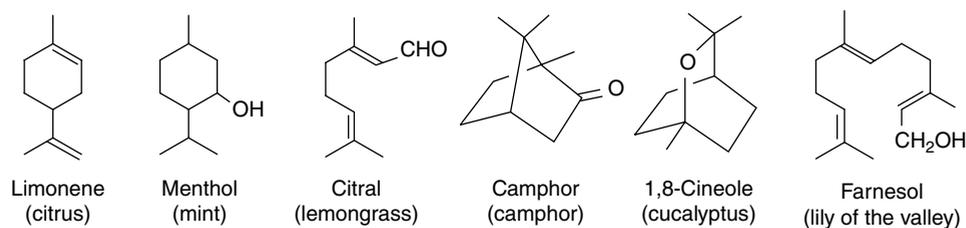


Figure 5.3 Key compounds in common essential oils.

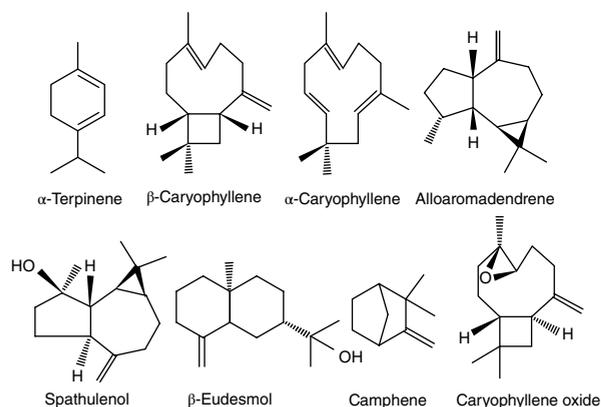


Figure 5.4 Compounds isolated from *M. tetramera* and *M. kwangsiensis* essential oils (Source: Chun-xue You *et al.*, 2015).

Table 5.1 Extraction of essential oils from *Murraya* (Source: Adapted from Chun-xue You *et al.*, 2015).

Species	Yield (V/W %)
<i>M. tetramera</i>	0.16
<i>M. euchrestifolia</i>	0.03
<i>M. koenigii</i>	0.23
<i>M. kwangsiensis</i>	0.10
<i>M. exotica</i>	0.12
<i>M. alata</i>	0.06

Solvent extraction due to a concrete, as a complex oil-soluble plant extract from the essential oils dispersed phase in solvent medium of dispersion, with some minor compounds such as waxes, resins, coumarin and vitamins. The extraction of absolute, as pure essential oil, from concrete is realised by liquid-liquid extraction with ethyl-alcohol, supercritical extraction and Freon or Florasol (R134a) cold extraction.

Table 5.2 Extraction yield and phenolic content of *C. violaceum* essential oil (Source: Hongmin Dong, 2015).

Samples ^A	Yield (%) ^B	Phenolic compounds (mg GAE/g) ^C
Ethanol	12.140 ± 1.112	7.879 ± 0.527 ^d
PEF	24.885 ± 1.667 ^b	51.723 ± 1.522 ^c
CF	16.763 ± 0.905 ^c	177.436 ± 5.968 ^b
EAF	1.928 ± 0.168 ^e	189.196 ± 4.944 ^a
BF	8.476 ± 0.387 ^d	44.653 ± 1.580 ^c
AF	47.949 ± 2.733 ^a	1.804 ± 0.141 ^d
Essential oil	0.083 ± 0.006	–
BHT	–	–
TBHQ	–	–
LSD (0.05)	2.596	7.183

All the values are mean ± SD; SD: standard deviation. –: not tested. A. PEF: petroleum ether fraction, CF: chloroform fraction, EAF: ethyl acetate fraction, BF: *n*-butanol fraction, AF: aqueous fraction. B. (a–e): different letters in the same column indicates significant differences ($p < 0.05$). c. mg GAE/g: mg of gallic acid equivalent per g of dry plant extract.

Gas chromatography (GC-MS) analysis is commonly performed in order to analyse the essential oil composition. The GC-MS investigation is commonly performed using a Thermo TRACE ultra gas chromatograph (GC) and an Thermo DSQ II mass analyser (MS). Also, NMR spectroscopy is a rapid method for essential oils compounds identification.

The principal natural compounds from the most known essential oils are presented in Tables 5.3 through 5.6.

The essential oils biological properties were investigated (Isman, 2006; Kordali *et al.*, 2008; Bakkali *et al.*, 2008; Regnault-Roger *et al.*, 2012) such as:

- insecticidal activity (Kumar *et al.*, 2011; Machial *et al.*, 2010; Maciel *et al.*, 2010; Liu *et al.*, 2010);
- repellent properties (Gu *et al.*, 2009; Zhang *et al.*, 2011; Mann *et al.*, 2012; Ukeh & Umoetok, 2011);
- lure (Dev *et al.*, 2010 & Kendra *et al.*, 2014);
- antifeedant capacity (Kumar *et al.*, 2009; Baskar & Ignacimuthu, 2012);
- antimicrobial activity (Smith-Palmer *et al.*, 1998; Seow *et al.*, 2014; Dhara & Tripathi, 2013).

The principal properties of organic bio compound from essential oils and their most common investigation methods are as follows:

5.1.1 Antioxidant Properties

Antioxidant activity of plant essential oil and extracts due to various applications in agro food industry and medicine: food preservation, pharmaceuticals, alternative medicine and natural therapies (Brahmi *et al.*, 2013; Šiler *et al.*, 2014; León *et al.*, 2014).

Table 5.3 *Anisomeles indica* essential oil composition (Source: G. Basappa *et al.*, 2015).

Compound	Rt. (min)	%
Unknown	4.81	10.68
Anisole	5.36	3.0
Acetoxy hexane	6.13	0.99
Benzaldehyde	6.42	–
Trimethyl benzaldehyde	20.79	0.11
Jasmatone	24.12	7.81
Apofarnesal-dihydro apofarnesal	28.77	0.96
Pygmaein	32.14	2.54
Cyclopentadecanolide	41.51	0.52
Farnesyl acetone	43.45	10.67
Nootkatone	50.11	8.35
Phytol acetate	54.03	7.35
Methyl communate	54.47	–
Unknown	66.44	5.59
Heptacosane	67.33	1.62
Unknown	73.63	4.05
Tritriacontane	80.40	3.63

Rt: retention time.

The antioxidant, antiinflammatory and antitumoral activities of the essential oil biocompounds create an important market of nutraceuticals to prevent various diseases (Franzini *et al.*, 2012; Huang *et al.*, 2013). The antioxidant activity might be determined by their complex phenolic structures represented by flavonoids, anthocyanins and coumarins, in principal (López-Mejía *et al.*, 2014; Witaicenis *et al.*, 2014). The sesquiterpenes give the antioxidant and free radical scavenging functions of the essential oils (Sharififar *et al.*, 2007).

The antioxidant activity of essential oils could be investigated with the following methods:

5.1.1.1 DPPH Assay

The free radical scavenging potential of essential oil is measured with the DPPH assay (Prakash *et al.*, 2010).

Different essential oil concentration (2.0–10.0 $\mu\text{L}/\text{mL}$) is added to 5 mL of 0.004% DPPH (w/v) in methanol and incubated for 30 min at the room temperature. BHA (butylated hydroxyanisole) 2.0–10.0 $\mu\text{g}/\text{mL}$ is used as positive control.

The antioxidant essential oil compounds determines the reducing of the absorbance value measured at the wavelength of 517 nm comparing with the control samples due to the scavenging effect of DPPH free radical.

Table 5.4 Chemical composition (%) of *Citrus aurantium* essential oil from peel (Source: Khaoula Zarrad *et al.*, 2015).

Compound	RI	RT	%
α -Pinene	949	4.097	0.561
Camphene	969	4.420	0.007
Sabinene	1001	4.998	0.171
β -Pinene	1007	5.080	0.453
b-Myrcene	1026	5.448	1.628
Octanal	1034	5.775	0.381
δ -3-Carene	1048	5.989	0.021
Limonene	1073	6.675	87.523
(<i>E</i>)- β -Ocimene	1086	7.224	0.325
γ -Terpinene	1109	7.568	0.045
Terpinolene	1141	8.660	0.215
Linalool	1152	9.141	3.365
Nonanal	1157	9.309	0.066
(<i>Z</i>)-Limonene oxide	1173	10.450	0.026
(<i>E</i>)-Limonene oxide	1182	10.643	0.022
Camphor	1192	10.870	0.021
Citronellal	1201	11.368	0.013
Terpinen-4-ol	1220	12.353	0.171
α -Terpineol	1231	12.982	0.928
Neral	1270	15.462	0.754
Geraniol	1280	16.157	0.222
Geranial	1291	16.896	0.430
Neryl acetate	1366	21.658	0.041
Geranyl acetate	1386	22.639	0.196
Caryophyllene	1420	23.988	0.111
Total		97.696	

RT: retention time. RI: retention indices calculated using an apolar column (HP-5).

The IC_{50} value is determined as ratio between volume and concentration of essential oil that neutralise 50% of DPPH radical, calculated using eqn. 5.1:

$$IC_{50\%} = \left(A_{\text{control}} - A_{\text{sample, 50\%}} / A_{\text{control}} \right) \times 100 \quad (\text{Eqn. 5.1})$$

Where, A_{control} is absorbance of control (without test compound), and $A_{\text{sample, 50\%}}$ is absorbance of test sample (Table 5.7).

Table 5.5 *C. violaceum* essential oil composition (Source: Dong, 2015).

RI ^a	Compounds name	Relative content (%)	Molecular formula
993	β-Myrcene	0.47	C ₁₀ H ₁₆
1005	Octanal	0.15	C ₈ H ₁₆ O
1033	γ-Terpinene	0.16	C ₁₀ H ₁₆
1049	β-Ocimene	0.12	C ₁₀ H ₁₆
1104	Nonanal	0.11	C ₉ H ₁₈ O
1130	1,2,3,4,5-Pentamethyl-1,3-cyclopentadiene	0.09	C ₁₀ H ₁₆
1201	α-Terpineol	0.27	C ₁₀ H ₁₈ O
1290	Bornyl acetate	0.18	C ₁₂ H ₂₀ O ₂
1424	Longifolene	0.13	C ₁₅ H ₂₄
1434	α-Cedrene	0.04	C ₁₅ H ₂₄
1485	α-Curcumene	0.58	C ₁₅ H ₂₂
1499	Geranyl isobutyrate	0.82	C ₁₄ H ₂₄ O ₂
1511	Nerol acetate	1.69	C ₁₂ H ₂₀ O ₂
1530	β-sesquiphellandrene	0.83	C ₁₅ H ₂₄
1544	(+)-α-Longipinene	0.59	C ₁₅ H ₂₄
1601	Neryl (S)-2-methylbutanoate	1.31	C ₁₂ H ₂₀ O ₂
1614	Cis-Z-α-Bisabolene epoxide	0.55	C ₁₅ H ₂₄ O
1626	2-(methylseleno) Acetaldehyde	1.82	C ₃ H ₆ OSe
1651	Geranyl tiglate	7.54	C ₁₅ H ₂₄ O ₂
1674	Isobornyl propionate	4.01	C ₁₃ H ₂₀ O ₂
1693	1,7-Dimethyl-4-(1-methylethyl)-Spiro[4.5]dec-6-en-8-one	9.08	C ₁₅ H ₂₄ O
1771	4-(1,5-Dimethyl-4-hexenyl)-2-cyclohexen-1-one	0.81	C ₁₄ H ₂₂ O
1872	Dibutyl phthalate	0.76	C ₁₆ H ₂₂ O ₄
1894	1-(1-Buten-3-yl)-2-vinyl-benzene	0.33	C ₁₂ H ₁₄
1922	Hexadecanoic acid, methyl ester	0.53	C ₁₇ H ₃₄ O ₂
1961	Hexadecanoic acid	1.70	C ₁₆ H ₃₂ O ₂
1990	Hexadecanoic acid, ethyl ester	0.35	C ₁₈ H ₃₆ O ₂
2094	9,12-Octadecadienoic acid (Z,Z)-,methyl ester	0.23	C ₁₉ H ₃₄ O ₂
2130	Falcarinol	57.02	C ₁₇ H ₂₄ O
2241	9,12-Octadecadienoic acid, ethyl ester	2.29	C ₂₀ H ₃₆ O ₂
Total identified		94.56	

^a Relative to C₇-C₃₀*n*-alknes determined using the HP-5MS capillary column.

Table 5.6 Cinnamon essential oil composition (Source: Yhang *et al.*, 2016).

Compounds	RI ^a	RT(min) ^b	PA(%) ^c
Styrene	1269	16.43	0.05
1-Methyl-2-isopropylbenzene	1279	16.79	0.01
Diacetone alcohol	1372	19.87	0.05
Benzaldehyde	1540	25.27	1.52
Butyric acid	1633	28.11	0.01
Benzylcarboxaldehyde	1660	28.89	0.10
Hexa-2,4-dienylbenzene	1797	32.81	0.09
trans-Cinnamaldehyde	1960	37.06	2.73
Cinnamaldehyde	2070	39.80	92.40
Phenol	2182	42.46	0.03
trans-Cinnamic acid	2249	43.97	0.25
(Z,Z)-9,12-Octadecadienoic acid	2311	45.35	0.66
E,E,Z-1,3,12-Nonadecatriene-5,14-diol	2390	47.04	0.06
			97.96

^a RI, retention index relative to *n*-alkane C₅ – C₂₈ on silica capillary column.

^b RT, retention time.

^c PA, peak area relative to the total oil peak area.

Table 5.7 *S. ringens* yield, total phenolic content (TPC), flavonoid content (FC) and antioxidant activities evaluated by DPPH, ABTS and FRAP assays (Source: Ana Alimpić *et al.*, 2015).

Sample	Yield ^a	TPC ^b	FC ^c	DPPH ^d	ABTS ^e	FRAP ^f
Methanol	6.94	185.05 ± 1.471	27.31 ± 0.588	20.29 ± 0.263	1.19 ± 0.026	274.85 ± 13.192
Dichloromethane	2.39	58.10 ± 0.510	32.31 ± 0.428	266.22 ± 4.208	0.58 ± 0.021	191.13 ± 11.020
Ethyl acetate	1.37	248.38 ± 0.455	66.67 ± 1.464	22.25 ± 0.571	2.36 ± 0.030	969.80 ± 25.238
Ethanol	7.20	208.27 ± 1.113	30.41 ± 0.64	17.26 ± 0.412	2.44 ± 0.028	1088.30 ± 17.655 ± 17.655
Essential oil	0.19	–	–	654.33 ± 6.522	nt	nt
BHA	–	–	–	17.94 ± 0.168	2.75 ± 0.021	445.34 ± 5.772
BHT	–	–	–	13.37 ± 0.430	2.82 ± 0.011	583.72 ± 5.255
Ascorbic acid	–	–	–	5.11 ± 0.143	–	180.81 ± 8.607

nt-not tested. a, Percentage of yield (%). b, mg GAE/g dry extract. c, mg QE/g dry extract. d, IC₅₀, µg/ml. e, mg AAE/g. f, µmol Fe(II)/g.

5.1.1.2 The Bleaching Assay

The mixture of essential oil different concentrations dissolved in DMSO and β-carotene/linoleic acid are thermo-stated two hours at a temperature of 50°C in the same time with the control sample. BHA is the positive control and DMSO is the negative control. Absorbance of the bleached and control samples is measured at the wavelength of 470 nm (Ebrahimabadi *et al.*, 2010).

Inhibition percentage ($I\%$) of essential oil could be determined using eqn. 5.2:

$$I\% = \left(A_{\beta\text{-carotene}, 2\text{h}} / A_{\beta\text{-carotene}, \text{initial}} \right) \times 100 \quad (\text{Eqn. 5.2})$$

Where,

$A_{\beta\text{-carotene}, 2\text{h}}$ is the absorbance due to the β -carotene remaining in sample after bleaching,

$A_{\beta\text{-carotene}, \text{initial}}$ is the absorbance due to the initial concentration of β -carotene from the control sample.

5.1.2 Anti-Microbial and Anti-Viral Activity

The essential oil prepared at different concentrations (0.1–2 $\mu\text{L} / \text{mL}$) in acetone are investigated for the minimum inhibitory concentration (MIC) evaluation of essential oil by considering the lowest concentration that did not allow any visible fungal, bacteria or virus growth (Burt, 2004).

5.1.3 Anti-Aflatoxic Activity

Anti-aflatoxic assay is carried out with essential oil samples added to the medium inoculated with 1 mL spore suspension (10^6 spores/mL) of aflatoxic strains. Cultures are incubated at $27 \pm 2^\circ\text{C}$ for 10 days, mycelia were collected by filtration, dried for 12 h at 80°C , subjected for aflatoxin extraction. The blue-coloured spots are collected and suspended into methanol (5 mL), centrifuged for 5 min at 3000 rpm, supernatants absorbance determined at 360 nm.

The anti-aflatoxic activity of the essential oil (AF) is quantified using eqn. 5.3 (Kumar *et al.*, 2007).

$$AF = (A \times MW / c \times PL) \times 1000 \quad (\text{Eqn. 5.3})$$

Where, A is absorbance, MW is molecular weight of AF , c is the molar extinction coefficient of AF and PL is path length (1 cm).

5.1.4 Anti-Inflammatory, Analgesic, Antipyretic, Pro-Kinetic and Pro-Immunity Activity (Pharmacologic Properties)

The acetic acid-inducing abdominal writhing movement test in mice response as abdominal constriction and the level of pain attenuate response in formalin-induced pain test could be used to investigate the analgesic activity of essential oils compounds. The anti-inflammatory activity could be performed using the carrageenan, dextran, histamine, serotonin, egg albumin or xylene-induced paw edemas model test. Antipyretic activity could be evaluated by yeast-induced hyperpyrexia in rats. The phytochemicals from essential oils sources (eucalyptus, lemongrass, *Manihot esculenta*, *Mangifera indica*, *Psidium guajava*, *Carica papaya* and *Citrus sinensis*), responsible for the complex and very effective pharmaceutical effects similar with classical drugs (aspirin, morphine, indomethacin, diclofenac and ibuprofen), were, in principal, tannins, flavonoids, glycosides, phlobatannins and antraquinones.

Essential oils from *Calendula officinalis* and *Melissa officinalis* prove a gastro-pro-kinetic property in biliary dysfunctions and slow or mal-digestion process.

The essential oils compounds act as pro-immunological agents because of antioxidant, anti-inflammatory anti antiseptic activity and, also, activate the welfare neuro-hormonal system and the pleasure area from the human brain.

Also, the positive effect in autoimmune and degenerative diseases or hormonal and emotional disorders is recently reported due to their softly and not entirely known mechanism of medical actions (Raut *et al.*, 2014).

5.1.5 Anti-Carcinogenic and Cytotoxicity Activity

The anti-carcinogenic activity of some essential oil compounds was reported in lymphatic, melanoma, breast or pancreatic carcinomas due to their complex pharmacological properties.

The proliferation rates of cells (of each cell line) in presence of essential oil could be determined by colourimetric MTT assay using 3-(4,5-dimethyl thiazol-2-yl) 2,5-diphenyl tetrazolium bromide (Mosmann, 1983). The mitochondrial dehydrogenases of viable cells determine the decreasing of the absorbance value at 570 nm in micro-plate reader (LISA Plus) assay correlated with the cell death test.

The IC₅₀ value is calculated as the concentration of essential oil with determine the decreasing of absorbance by 50% in treated with essential oils samples compared with the untreated samples. The cell viability percentage is determined using eqn. 5.1.

The essential oils cytotoxicity, tested on specific microorganisms and on human cells, is presented in Table 5.8, Table 5.9 and Table 5.10, respectively.

Also, the anti-plasmodial activity with anti-parasitic and anti-malarial effect in case of *Acanthospermum hispidum*, *Dicoma tomentosa* and *Sorindeia juglandifolia* essential oils is usually investigated *in vitro* as inhibitory activity (plasmodial lactate dehydrogenase activity) against chloroquine-sensitive strain of *Plasmodium falciparum* (3D7) and chloroquine-resistant Dd2 and *in vivo* by suppressive test against *Plasmodium berghei berghei* (Wells, 2011).

5.2 Final Conclusions

Essential oils have complex and unique chemical composition and the biochemical mechanism of action on the pathogens or spoilage organisms is specific for each key-compound, a synergistic or antagonist effect could occur between the active components from the natural mixture.

The essential oils active compounds responsible for the antioxidant, anti-mycobacterial, anti-viral or anti-carcinogenic (anti-tumoral) activities has molecular characteristics (unipolarity, the hydrophobicity, the molar volume and the dipole moment) associated with the specific biological activity (cellular and metabolism damage of the bio target site), correlation reported in the recent studies using the quantitative structure-activity relationship (QSAR) model (Andrade-Ochoa *et al.*, 2015).

Linalool, geraniol and menthol are the three most effective compounds from essential oils composition, with the largest range of antibacterial and antifungal action. The biomembrane permeability, polarity and integrity modification (cytotoxicity), mitochondrial and

Table 5.8 Essential oils cytotoxicity (Source: Adapted from Bakkali *et al.*, 2008).

Essential oil or components	Target Organisms	Cytotoxicity concentrations	Selected references
<i>Pinus densiflora</i> <i>Pinus koraiensis</i> <i>Chamaecyparis obtusa</i>	<i>Salmonella typhimurium</i> <i>Listeria monocytogenes</i> <i>Escherichia coli</i> <i>Staphylococcus aureus</i> <i>Klebsiella pneumoniae</i> <i>Candida albicans</i>	50 µL of 1/2, 1/4, 1/8, 1/16 diluted solutions	Hong <i>et al.</i> (2004)
<i>Melissa officinalis</i>	<i>Escherichia coli</i> <i>Salmonella</i> <i>Micrococcus flavus</i> <i>Staphylococcus</i> <i>Bacillus subtilis</i> <i>Candida albicans</i>	20%, 50% MIC 15–30 µL/mL	Mimica-Dukic <i>et al.</i> (2004)
<i>Rosmarinus officinalis</i> <i>Lavandula latifolia</i> <i>Lavandula angustifolia</i> <i>Thymus vulgaris</i> <i>Salvia sclarea</i> <i>Salvia officinalis</i> <i>Salvia lavandulifolia</i>	<i>Escherichia coli</i> <i>Listeria monocytogenes</i> <i>Staphylococcus aureus</i> <i>Salmonella enteritidis</i> <i>Salmonella typhimurium</i> <i>Yersinia enterocolitica</i> <i>Shigella flexneri</i>	MIC < 0.1–5.0 µL/mL	Rota <i>et al.</i> (2004)
<i>Salvia sclarea</i> Linalyl acetate Linalool	<i>Sclerotinia sclerotiorum</i> <i>Sclerotium cepivorum</i> <i>Fusarium oxysporum</i>	EC50 492.55 µL/L EC50 544.17 µL/L EC50 584.36 µL/L EC50 549.62 µL/L EC50 > 1500 µL/L EC50 > 1500 µL/L EC50 146.15 µL/L EC50 563.94 µL/L EC50 661.76 µL/L	Pitarokili <i>et al.</i> (2002)
<i>Salvia fruticosa</i> 1,8-cineole Camphor	<i>Fusarium oxysporum</i> <i>Fusarium solani</i> <i>Fusarium proliferatum</i> <i>Sclerotinia sclerotiorum</i> <i>Rhizoclonia solani</i>	50–2000 µL/L 20–500 µL/L MIC > or = 2000 µL/L MIC > 500 µL/L	Pitarokili <i>et al.</i> (2003)
<i>Salvia desoleana</i> <i>Salvia sclarea</i> α-Terpineol Linalool	<i>Staphylococcus aureus</i> <i>Staphylococcus epidermidis</i> <i>Escherichia coli</i> <i>Candida albicans</i> <i>Pseudomonas aeruginosa</i>	MIC 2 or >2 mg/mL MIC 1.5–2 mg/mL MIC 0.250–1 or >2 mg/mL MIC 1–2 or >2 mg/mL	Peana <i>et al.</i> (1999)

Table 5.8 (Continued)

Essential oil or components	Target Organisms	Cytotoxicity concentrations	Selected references
<i>Grammosciadium platycarpum</i>	<i>Bacillus subtilis</i>	MIC 0.5–1.9 mg/mL	Sonboli <i>et al.</i> (2005)
Linalool	<i>Enterococcus faecalis</i>	MIC 7.5–15 mg/mL	
Limonene	<i>Staphylococcus aureus</i>	MIC 0.2–2.5 mg/mL	
	<i>Staphylococcus epidermidis</i>	MIC 0.6–5 mg/mL	
	<i>Escherichia coli</i>		
	<i>Pseudomonas aeruginosa</i>		
	<i>Klebsiella pneumoniae</i>		
<i>Ziziphora clinopodioides</i>	<i>Staphylococcus epidermidis</i>	10 µL/filter paper disc	Sonboli <i>et al.</i> (2006)
Pulegone	<i>Staphylococcus aureus</i>	MIC 3.75 to >15 mg/mL	
1,8-cineole	<i>Staphylococcus aureus</i>	MIC 1.8–7.2 mg/mL	
	<i>Escherichia coli</i>	MIC 0.9–7.2 mg/mL	
	<i>Bacillus subtilis</i>		
	<i>Enterococcus faecalis</i>		
	<i>Klebsiella pneumoniae</i>		
	<i>Pseudomonas aeruginosa</i>		
<i>Pimpinella anisum</i>	<i>Candida albicans</i>	MIC 0.1–1.56% v/v	Kosalec <i>et al.</i> (2005)
	<i>Candida tropicalis</i>		
	<i>Trichophyton rubrum</i>		
	<i>Trichophyton mentagrophytes</i>		
	<i>Microsporium canis</i>		
<i>Origanum</i>	<i>Candida albicans</i>	0.0625, 0.125,	Manohar <i>et al.</i> (2001)
Carvacrol		0.25 mg/mL	
<i>Calamintha officinalis</i> , <i>Lavandula dentata</i> , <i>Mentha pulegium</i> , <i>Origanum compactum</i> , <i>Rosmarinus officinalis</i> , <i>Salvia aegyptiaca</i> , <i>Thymus glandulosus</i> , α-Pinene, borneol, thymol, Carvacrol, cineole, p-cimene, Linalool, menthone, R-(+)-pulegone	<i>Botrytis cinerea</i>	10–250 ppm	Bouchra <i>et al.</i> (2003)
Thyme, basil, thymol, estragol, linalool, carvacrol	<i>Shigella sonnei</i>	0.1–10%	Bagamboula <i>et al.</i> (2004)
	<i>Shigella flexneri</i>	0.05% (lettuce)	
	<i>Escherichia coli</i>		
<i>Coriandrum sativum</i>	<i>Salmonella choleraesuis</i>	6.25 µg/mL 12.5 µg/mL	Kubo <i>et al.</i> (2004)

(Continued)

Table 5.8 (Continued)

Essential oil or components	Target Organisms	Cytotoxicity concentrations	Selected references
<i>Anethum graveolens</i>	<i>Escherichia coli</i>	MIC 0.02–0.10–0.47% v/v	Delaquis <i>et al.</i> (2002)
<i>Coriandrum sativum</i> (seeds)	<i>Salmonella typhimurium</i>	MIC 0.02–0.10–0.47% v/v	
<i>Coriandrum sativum</i> (leaves)	<i>Listeria monocytogenes</i>	MIC 0.01–0.10–0.47% v/v	
<i>Eucalyptus dives</i> and fractions	<i>Staphylococcus aureus</i>	MIC 0.04–0.13–0.43% v/v	
	<i>Pseudomonas fragi</i>		
	<i>Serratia grimesii</i>		
	<i>Enterobacter agglomerans</i>		
	<i>Yersinia enterocolitica</i>		
	<i>Bacillus cereus</i>		
	Group A <i>Streptococcus</i>		
	<i>Lactobacillus</i>		
	<i>Saccharomyces cerevisiae</i>		
	Carvone	<i>Penicillium citrinum</i>	IC50 5, 2 µg/mL
Piperitone (<i>Artemisia herba-alba</i>)	<i>Mucora rouxii</i>	IC50 7, 1.5 µg/mL	
<i>Anthemis aciphylla</i>	<i>Staphylococcus aureus</i>	0.06–1.0 mg/mL	Hüsni Can Baser <i>et al.</i> (2006)
	<i>Pseudomonas aeruginosa</i>		
	<i>Enterobacter aerogenes</i>		
	<i>Staphylococcus epidermidis</i>		
	<i>Salmonella typhimurium</i>		
	<i>Candida albicans</i>		
<i>Lippia sidoides</i>	<i>Streptococcus</i>	MIC 0.625–10 mg/mL	Botelho <i>et al.</i> (2007)
	<i>Candida albicans</i>		
Thymol, carvacrol			
<i>Myrtus communis</i>	<i>Bacillus cereus</i>	MIC 1.4–11.20 mg/ mL	Rosato <i>et al.</i> (2007)
<i>Origanum vulgare</i>	<i>Bacillus subtilis</i>	MIC 0.35–0.70 mg/mL	
<i>Pelargonium graveolens</i>	<i>Escherichia coli</i>	MIC 0.36–5.60 mg/mL	
<i>Rosmarinus officinalis</i>	<i>Staphylococcus aureus</i>	MIC 1.40–11.20 mg/mL	
<i>Salvia officinalis</i>		MIC 1.40–11.20 mg/mL	
<i>Thymus serpyllum</i>		MIC 0.28–1.40 mg/mL	
Citronellol		MIC 0.35–1.40 mg/mL	
Eucalyptol		MIC 2.80–5.60 mg/mL	
Geraniol		MIC 0.08–1.40 mg/mL	
Thymol		MIC 0.7–1.40 mg/mL	
Carvacrol		MIC 0.35–2.80 mg/mL	
Triacetin		MIC 22.40 mg/mL	

EC: effective concentration, MIC: minimum inhibitory concentration, IC: inhibitory concentration.

Table 5.9 Cinnamon essential oil bactericide characteristics (Source: Yhang *et al.*, 2016).

Microorganisms	DIZ (mm) ^a	MIC (mg/ml) ^a	MBC (mg/ml) ^c
<i>E. coli</i>	19.2 ± 1.3 ^b	1.0	4.0
<i>S. aureus</i>	28.7 ± 2.0 ^a	1.0	2.0

^a DIZ, diameter of inhibition zone, values represent mean of three replicates ± SD, including diameter of disc 6mm EO. Different letters within the same row express significantly differences between the means ($P < 0.05$).

^b MIC, minimal inhibitory concentration.

^c MBC, minimal bactericide concentration.

Table 5.10 *Artemisia Vulgaris L.* essential oil IC₅₀ values in human cell lines (Source: Saleh *et al.*, 2014).

Cell line	Origin	IC50 µg/mL
MCF-7	Human breast	1.00
HepG2	Human liver	0.72
PC-3	Human prostate	0.74
HeLa	Human cervical	2.50
BJ	Human skin	17.75
HEK-293	Human kidney	13.24

genomic DNA damage (genotoxicity) or loss of electrolytes was cited as causes for the antimicrobial and carcinogenic effect.

Efficient uses of optimal essential oils doses could attempt the desire single or synergetic effect as antioxidant, anti-inflammatory, analgesic, antimicrobial and anti-mutagenic agent in food preservation, plant protection and human classic or holistic medicine, without any risks of side effects due to the cytotoxicity or genotoxicity of the overdoses, targeted on human or nature.

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6

Antimicrobial Activity of Essential Oil

Saeedeh Shojaee-Aliabadi, Seyede Marzieh Hosseini and Liela Mirmoghtadaie

Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

6.1 Chemical Composition of Essential Oils

Essential oils are natural mixture of volatile compounds that produced by approximately 60 families of plants (Raut & Karuppaiyil, 2014). These complex mixtures can be extracted from different parts of plant that have effect on chemical composition of essential oil (Dorman & Deans, 2000). There are about 20 to 60 effective components at different concentrations in essential oils. Generally, essential oils characterized by some major components that present at high concentration (20–70%) and other components are in trace amounts. Biological effects of essential oils such as antimicrobial activity mainly depend on these major components (Bakkali *et al.*, 2008). However, antimicrobial activity of EOs can't be due to only the action one compound and certainly their chemical composition are the most important factor for their antimicrobial efficacy. For example, fractions that have more long chain alcohol and aldehydes were more active against gram-positive bacteria. Because it is known that the antimicrobial activity of alcohols increase with increasing of molecular weight (Delaquis *et al.*, 2002).

Generally, according to biosynthetic origin, the components of EOs are divided in two groups, including terpene origin compounds and aromatic constitutes (Bakkali *et al.*, 2008, Perricone *et al.*, 2015).

6.1.1 Terpene Origin Compounds

Terpene origin compounds defined as substances composed of 5-carbon-base (C₅) units (2-methylbutadiene) that called isoprene. Terpenes are biosynthesised in different processes including:

- The isopentenyl diphosphate (IPP) precursor synthesis;
- Formation of the prenyldiphosphate by repetitive addition of IPPs that is precursor of terpenes;
- Formation of terpene skeleton by modification of the allylic prenyldiphosphate;

- Redox reaction of the skeleton (secondary enzymatic modification) to ascribe functional properties to the different terpenes (19). The terpenes compounds exist in the monoterpenes (C10), sesquiterpenes (C15), hemiterpenes (C5), diterpenes (C20), triterpenes (C30) and tetraterpenes (C40). The monoterpenes are the major components of most of all the essential oils. It is known that monoterpenes has the membrane-damaging effects on microorganism; as they diffuse into the cell membrane structures and damage them (Hyldgaard *et al.*, 2012). In addition, monoterpenes containing oxygen (ketone, alcohol, ether or aldehyde forms) have more antifungal activity than monoterpenes hydrocarbons (Ye *et al.*, 2013). Different types of monoterpenes are listed in Table 6.1 (Bakkali *et al.*, 2008; Baser & Buchbauer, 2009).

The sesquiterpenoids contain three isoprene units (15 carbon atoms). As a result, in comparison to monoterpenoids, they have lower volatilities and therefore, have a lesser role in odour of essential oils. Furthermore, they act as fixatives for more volatile compounds. Farnesol is the sesquiterpenoids precursor, whereas geraniol is

Table 6.1 Different types of monoterpenes and sesquiterpenes (Source: Bakkali *et al.*, 2008).

Components	Monoterpenes	Sesquiterpenes
Carbures	Acyclic structures: myrcene, α , β and allo ocimene, <i>etc.</i> Monocyclic structure: <i>terpinenes, p-cimene,</i> <i>phellandrenes, etc.</i> Bicyclic structure: pinenes, -3-carene, camphene, sabinene, <i>etc.</i>	Guaiazulene, b-bisabolene, cadinenes, b-caryophyllene, longifolene, curcumenes, elemenes, farnesenes, zingiberene, <i>etc.</i>
Alcohols	Acyclic structures: geraniol, linalool, citronellol, lavandulol, nerol, <i>etc.</i> Monocyclic structures: menthol, α -terpineol, terpinen-4-ol, carveol, Isopulegol Bicyclic structures: borneol, fenchol, chrysanthenol, thuyen-3-ol, <i>etc.</i>	bisabol, cedrol, b-nerolidol, farnesol, carotol, elemol b-santalol, patchoulol, viridiflorol, <i>etc.</i>
Ketones	Acyclic structures: tegetone, <i>etc.</i> Monocyclic structures: menthones, carvone, pulegone, piperitone, <i>etc.</i> Bicyclic structures: camphor, fenchone, thuyone, ombellulone, pinocamphone, pinocarvone, <i>etc.</i>	Acorane, germacrone, nootkatone, cis-longipinan-2,7- dione, b-vetinone, turmerones, <i>etc.</i>
Aldehydes	geranial, neral, citronellal, compholenic aldehyde, <i>etc.</i>	–
Ethers	1,8-cineole, menthofurane, <i>etc.</i>	–
Peroxides	ascaridole, <i>etc.</i>	–
Phenols	thymol, carvacrol, <i>etc.</i>	–
Epoxyds	–	caryophyllene oxide, humulene epoxides, <i>etc.</i>

the precursor for the monoterpenoids (Baser & Buchbauer, 2009). However, the functional properties of sesquiterpenoids are very close to monoterpenoids compounds. Different structures of sesquiterpenoids also presented in Table 6.1 (Bakkali *et al.*, 2008).

6.1.2 Aromatic Compounds

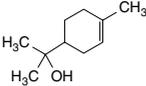
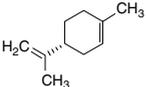
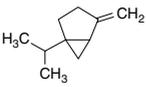
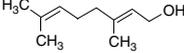
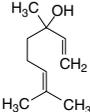
Aromatic compounds are the other chemical class of constituents in essential oils, derived from phenylpropane, which occurred less frequently than the terpenoids compounds (Bakkali *et al.*, 2008; Yazaki *et al.*, 2009). These components comprise aldehydes (such as cinnamaldehyde), alcohols (such as cinnamic alcohol), phenols (like chavicol, eugenol), methoxy derivatives (such as anethole, elemicine, estragole, methyleugenols), and methylene dioxy compounds (like apiole, myristicine, safrole).

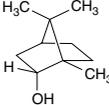
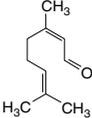
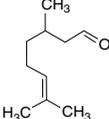
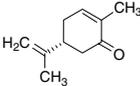
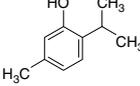
Furthermore, there are other various chemical components present in essential oils. For example, nitrogen and sulphur components such as glucosinolates or isothiocyanate derivatives (garlic and mustard oils) are also characterized as plant-secondary metabolites (Bakkali *et al.*, 2008). Table 6.2 present chemical structures of some selected components of essential oils with their essential oils sources and minimum inhibitory concentration (MIC).

6.2 Antimicrobial Activity of Essential Oils

Essential oils have antimicrobial activity against a broad spectrum of gram-positive and gram-negative bacteria. One of the first *in vitro* studies on the antimicrobial activity of essential oils was done by Buchholtz in 1875. This author investigated the inhibitory activity of caraway oil, thyme oil, phenol and thymol on bacteria cultivated in a tabac decoction and found that thymol had stronger antibacterial activity than phenol (Baser & Buchbauer, 2009). Subba *et al.* (1967) reported inhibitory activity of orange and lemon oil on a wide range of spoilages bacteria (Subba *et al.*, 1967). Deans & Ritchie (1987) examined antibacterial properties of 50 essential oils against 25 genera of bacteria. In this study, thyme, cinnamon, bay, clove, almond, lovage, pimento, marjoram, angelica and nutmeg were reported as the 10 most inhibitory essential oils. Furthermore, they found no difference between sensitivity of gram-negative and gram-positive organisms (Deans & Ritchie, 1987). There have been many studies in this area. The antibacterial activity of essential oils varies according to the type of bacteria as well as with the type of essential oils. In general, the gram-positive bacteria are more susceptible to antimicrobial compounds such as essential oils than to gram-negative bacteria that may be related to the lack of outer membrane in gram-positive organisms. However, gram-negative bacteria have been reported to be more sensitive against EOs in some other studies. For example, it seems that *Aeromonas hydrophila* is one of the most sensitive bacteria (Burt, 2004). Oregano, rose geranium, black pepper, clove, thyme and *Thymus* spp. essential oils antimicrobial activities against this bacteria have been reported (Raut & Karuppayil, 2014). Another study showed more sensitivity of gram-negative organisms to mint essential oils that were higher for *Salmonella enteritidis* than *Listeria monocytogenes* in the Greek appetisers taramosalata and tzatziki (Tassou *et al.*, 1995). In addition, some studies have also pointed to the lack of

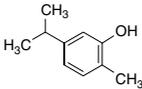
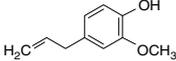
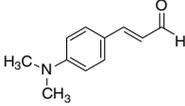
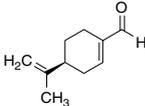
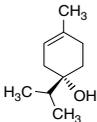
Table 6.2 Chemical structures of some selected components of EOs with antimicrobial activity.

Component	EOs sources	MIC ($\mu\text{g/ml}$)	Structural formulae	References
α -terpineol	Cryptomeria japonica, green tea	<i>E. coli</i> : 1600 <i>S. aureus</i> : 1600 <i>Streptococcus</i> spp.: 50–3200		(Cha <i>et al.</i> , 2007; Llana-Ruiz-Cabello <i>et al.</i> , 2015)
D-Limonène	citrus-derived essential oils such as orange, lemon, mandarin, lime, grapefruit)	<i>S. aureus</i> : 1 <i>B. subtilis</i> : 1 <i>E. coli</i> : 1 <i>S. cerevisiae</i> : 0.5		(Zhang <i>et al.</i> , 2014)
Sabinene	Cryptomeria japonica, carrot (juice)	<i>E. coli</i> : 1600 <i>S. aureus</i> : 1600 <i>Streptococcus</i> spp.: 100–1600		(Cha <i>et al.</i> , 2007; Calo <i>et al.</i> , 2015)
Geraniol	Palmarosa (<i>Cymbopogon martini</i>),	<i>E. coli</i> : 0.5 <i>S. typhimurium</i> : 0.5 <i>L. monocytogenes</i> : 1		(Kim <i>et al.</i> , 1995; Llana-Ruiz-Cabello <i>et al.</i> , 2015)
Linalool	Basil, cilantro, green tea	<i>Streptococcus</i> spp.: 100–3200		(Park <i>et al.</i> , 2012; Llana-Ruiz-Cabello <i>et al.</i> , 2015)

d- Borneol	<i>Cinnamomum camphora</i>	<i>P. aeruginosa</i> : 125 <i>E. coli</i> : 250 <i>B. subtilis</i> : 125 <i>S. aureus</i> : 125 <i>H. anomala</i> : 250 <i>S. cescerevisiae</i> : 125 <i>A. niger</i> : 250 <i>C. globosum</i> : 62.5		(Su <i>et al.</i> , 2012)
Citral	Lemongrass, citrus peel	<i>E. coli</i> : 0.5 <i>S. typhimurium</i> : 0.5 <i>S. aureus</i> : 0.5 <i>L. monocytogenes</i> : 0.5		(Burt, 2004; Perricone <i>et al.</i> , 2015; Llana-Ruiz-Cabello <i>et al.</i> , 2015)
Citronellal	<i>Cymbopogon nardus</i> ; <i>Corymbia citriodoura</i>	<i>Rhizoctonia solani</i> : 5 <i>Helminthosporium oryzae</i> : 2.5 <i>Aspergillus</i> spp.: 25 <i>Pyricularia grisea</i> : 50 <i>Colletotrichum musae</i> : 25		(Ramezani <i>et al.</i> , 2002; Aguiar <i>et al.</i> , 2014)
Carvone	<i>Ocimum sanctum</i>	<i>Candida</i> spp.: 250–350		(Amber <i>et al.</i> , 2010)
Thymol	Oregano, thyme	<i>E. coli</i> : 0.225–0.45 <i>S. typhimurium</i> : 0.056 <i>S. aureus</i> : 0.140–0.225 <i>L. monocytogenes</i> : 0.450 <i>B. cereus</i> : 0.450		(Lambert <i>et al.</i> , 2001; Cosentino <i>et al.</i> , 1999; Burt, 2004)

(Continued)

Table 6.2 (Continued)

Component	EOs sources	MIC ($\mu\text{g/ml}$)	Structural formulae	References
Carvacrol	Oregano, thyme	<i>E. coli</i> : 0.225–5 <i>S. typhimurium</i> : 0.225–0.25 <i>S. aureus</i> : 0.175–0.450 <i>L. monocytogenes</i> : 0.375–5		(Lambert <i>et al.</i> , 2001; Kim <i>et al.</i> , 1995; Burt, 2004)
Eugenol	Clove, cinnamon	<i>E. coli</i> : 1.0 <i>S. typhimurium</i> : 0.5 <i>L. monocytogenes</i> : >1.0		(Kim <i>et al.</i> , 1995; Llana-Ruiz-Cabello <i>et al.</i> , 2015)
Cinnamaldehyde	Cinnamon	Bacteria (<i>E. coli</i> , <i>Enterobacter aerogenes</i> , <i>Proteus vulgaris</i> , <i>P. aeruginosa</i> , <i>Vibrio SPP</i> , <i>S. typhimurium</i>): 75–600 $\mu\text{g/ml}$, <i>Candida</i> spp.: 100–450 $\mu\text{g/ml}$, filamentous fungi (<i>Aspergillus</i> spp. and one <i>Fusarium</i> spp.): 75–150 $\mu\text{g/ml}$		(Ooi <i>et al.</i> , 2006)
Perillaldehyde	Perilla	<i>E. coli</i> : 1.0 <i>S. typhimurium</i> : 0.5 <i>L. monocytogenes</i> : 1.0		(Kim <i>et al.</i> , 1995; Burt, 2004)
Terpinen-4-ol	Cryptomeria japonica	<i>E. coli</i> : 1600 <i>S. aureus</i> : 1600 <i>Streptococcus</i> spp.: 50–3200		(Cha <i>et al.</i> , 2007)

difference between these two groups of organisms (Deans & Ritchie, 1987; Ouattara *et al.*, 1997). *Pseudomonas aeruginosa* has shown tolerance to inhibition by essential oils in comparison to other bacteria. Generally, oregano, thyme, cinnamon, lemon grass, tea-tree, clove, rosewood, bay and lemon-myrtle oils are the most active antimicrobials. So that they could act as antimicrobial agents at concentration <1% v/v and their minimum concentration for inhibition is lower than 1% (Hammer *et al.*, 1999). Oregano is one of the most valued spices commonly used in world. More than 60 plant species are used as oregano (Baser & Buchbauer, 2009). Major volatile components of oregano spices essential oils are sabinyl monoterpenes, terpinen-4-ol, γ -terpinene, carvacrol and thymol. Antimicrobial activity of these essential oils against several foodborne pathogens including *B. cereus*, *B. subtilis*, *C. botulinum*, *E. faecalis*, *E. coli*, *S. aureus*, *L. monocytogenes*, *K. pneumoniae*, *Ps. aeruginosa* and *Salmonella* spp. have been reported (Perricone *et al.*, 2015). In thyme EO groups, commonly, thymol, *p*-cymene, γ -terpinene and linalool are the active main components that have shown inhibitory effect against *B. cereus*, *botulinum*, *E. faecalis*, *E. coli*, *S. aureus*, *L. monocytogenes*, *K. pneumoniae*, *Ps. aeruginosa*, *Salmonella* et Cinnamaldehyde, eugenol, copaene and β -caryophyllene are the main active components of cinnamon essential oils that are active against *B. cereus*, *E. coli*, *Shigella* spp., *Vibrio parahaemolyticus*, *Yersinia enterocolitica*, *Salmonella enterica* serovars *Enteritidis*, *Infantis*, *Typhimurium*, *B. subtilis*, *E. faecalis* and so on. In clove essential oils, eugenol, eugenyl acetate and caryophyllene are the major volatile constituents and their antimicrobial properties against *B. brevis*, *B. subtilis*, *Cl. botulinum*, *E. faecalis*, *Candida* spp., *E. coli*, *K. pneumoniae*, *Ps. aeruginosa*, *S. aureus*, *Salmonella* spp. and *L. monocytogenes* have been pointed out (Perricone *et al.*, 2015).

It has been reported that 60% of plant essential oils derivatives have inhibitory effect against large variety of fungi that could be used in food and medicine industries (Bertoli *et al.*, 2011). Antifungal activity might be depended on the physiological activity of target organisms and the tested essential oils (Daferera *et al.*, 2000). The antifungal efficacy of essential oils can be related to the presence of some active components such as carvacrol, α -terpinyl acetate, cymene, thymol, pinene and linalool which are also known as active components in antimicrobial properties of essential oils (Bertoli *et al.*, 2011; Nuzhat & Vidyasagar, 2014). Another typical component of essential oils that has antifungal activity, is CHO group of unsaturated aldehydes, when conjugated with a carbon in the form of C=C (Moleyar & Narasimham, 1986). Some of most important fungal pathogens that inhibited by essential oils are *Aspergillus* spp., *Candida* spp., *Botrytis* spp., *Penicillium* spp., *Fusarium* spp., *Geotrichum candidum*, *Rhizocotonia solani*, *Macrophomina* spp., *Microsporum* spp., *Mucor ramannianus* and *Trichophyton* spp. The reported effective essential oils against foodborne spices of *Aspergillus*, including *Aspergillus niger*, *A. flavus*, and *A. parasiticus* and *A. fumigatus* are garlic, wormwood, black caraway, cedar wood oil, *Chenopodium ambrosioides*, ginger grass, lemon grass, clove, fennel, juniper, chamomile, *Tamarix boveana*, *Satureja hortensis* (summer savoury), rosemary, cumin, black cumin, *Artemisia* spp. such as *A. absinthium*, *A. biennis* and others. *Botrytis* spp. such as *B. cinerea* and *B. fabae* were inhibited by rosemary, fennel, wormwood, *Artemisia absinthium*, *A. biennis* and other *Artemisia* spp. (Raut & Karuppayil, 2014).

Antimicrobial activities of the most important plant families producing essential oils are shown in Table 6.3.

Table 6.3 Antimicrobial activities of the most important plant families producing EOs.

Plant family	Common name	Species	Part of plant	Target organisms	References
I. Apiaceae	Angelica	<i>Angelica archangelica</i>	Root	<i>Clostridium difficile</i> , <i>perfringens</i> , <i>E. faecalis</i> , <i>Eubacterium limosum</i> , <i>Peptostreptococcus anaerobius</i> , and <i>Candida albicans</i>	(Fraternali <i>et al.</i> , 2014)
	Anise	<i>Pimpinella anisum</i> L.	Seed/ Fruit	<i>E. coli</i> ; <i>Candida</i> spp.; <i>Geotrichum</i> spp.; <i>Trichophyton</i> spp.; <i>Aspergillus parasiticus</i> , <i>A. Niger</i> ; <i>Alternaria. alterna</i>	(Gülçın <i>et al.</i> , 2003; Kosalec <i>et al.</i> , 2005; Özcan & Chalchat, 2006)
	Black caraway	<i>Carum nigrum</i>	Seed	<i>B. cereus</i> ; <i>Pseudomonas aeruginosa</i> ; <i>Aspergillus niger</i> ; <i>A. flavus</i> ; <i>Penicillium</i> spp.;	(Singh <i>et al.</i> , 2006; Raut & Karuppayil, 2014)
	Carrot seed	<i>Daucus carota</i>	Seed	<i>Aspergillus</i> spp.; <i>dermatophyte</i> ; <i>yeasts</i>	(Tavares <i>et al.</i> , 2008)
	Celery seed	<i>Apium graveolens</i>	Seed	<i>E. coli</i> ; <i>Pseudomonas aeruginosa</i> ; <i>S. aureus</i> ;	(Dorman & Deans, 2000; Hammer <i>et al.</i> , 1999)
	Coriander	<i>Coriandrum sativum</i>	Fruit	<i>Salmonella typhimurium</i> ; <i>L. monocytogenes</i> ;	(Delaquis <i>et al.</i> , 2002)
	Cumin	<i>Cuminum cyminum</i>	Fruit	<i>A. Flavus</i> ; <i>A. fumigatus</i> ;	(Kedia <i>et al.</i> , 2014)
	Dill	<i>Anethum graveolens</i> and <i>A. sowa</i> Roxb	Fruit	<i>E. coli</i> ; <i>Klebsiella pneumonia</i> ; <i>L. monocytogenes</i> ; <i>Pseudomonas aeruginosa</i> ; <i>Aspergillus niger</i> ; <i>Saccharomyces cerevisiae</i> ; <i>Candida albicans</i> .	(Dorman & Deans, 2000; Delaquis <i>et al.</i> , 2002; Kaur & Arora, 2009; Jirovetz <i>et al.</i> , 2003)
	Fennel	<i>Foeniculum vulgare</i>	Fruit	<i>Alternaria alternate</i> ; <i>Aspergillus niger</i> ; <i>A. fumigatus</i> ; <i>Botrytis</i> spp.; <i>E. coli</i> O157:H7; <i>L. monocytogenes</i> ; <i>Salmonella typhimurium</i> ; and <i>S. aureus</i>	(Shahat <i>et al.</i> , 2011; Sridhar <i>et al.</i> , 2003; Dadalioglu & Evrendilek, 2004)
	Galbanum	<i>Ferula galbaniflua</i> Boiss. <i>F. rubricaulis</i> Boiss	Fruit	<i>S. aureus</i> ; <i>Bacillus cereus</i>	(Asili <i>et al.</i> , 2009)

	Lovage root	<i>Levisticum officinale</i>	Root	<i>Albicans; S. aureus; B. subtilis</i>	(Janssen <i>et al.</i> , 1986)
	Parsley	<i>Petroselinum crispum</i>	Seed	<i>Albicans; S. aureus; B. subtilis</i>	(Janssen <i>et al.</i> , 1986)
2. Araceae	Calamus	<i>Acorus calamus</i>	Rhizome/ leaf	<i>Albicans; S. aureus; B. subtilis;</i>	(Janssen <i>et al.</i> , 1986)
3. Annonaceae	Cananga (ylang ylang)	<i>Cananga odourata</i> hook	Flower	<i>Albicans; S. aureus; B. subtilis; Acinetobacter baumannii; E. faecalis; E. coli;</i>	(Janssen <i>et al.</i> , 1986; Hammer <i>et al.</i> , 1999)
4. Asteraceae	Chamomile	<i>Matricaria chamomilla</i>	Flower	<i>Aspergillus niger; A. flavus; C. albicans; B. cereus; S. aureus</i>	(Pawar & Thaker, 2006; Roby <i>et al.</i> , 2013)
	Davana	<i>Artemisia pallens</i>	Flowering Herb	<i>B. cereus</i>	(Ruikar <i>et al.</i> , 2009)
	Tarragon	<i>Artemisia dracunculus</i>	Herb	<i>E. coli; S. aureus; S. epidermidis; albicans; Cryptococcus. Neoformans; dermatophytes Trichophyton, Microsporum spp.; Fonsecaea pedrosoi; A. niger</i>	(Lopes-Lutz <i>et al.</i> , 2008)
	Wormwood	<i>Artemisia absinthium</i>	Herb	<i>Aspergillus niger; A. fumigatus; Candida spp.; Botrytis spp.; Penicillium spp.; Fusarium spp.;</i>	(Mohamed <i>et al.</i> , 2010; Raut & Karuppaiyl, 2014)
5. Alliaceae	Garlic	<i>Allium sativum</i>	Bulb	<i>Aspergillus niger; Penicillium spp.; Fusarium spp.;</i>	(Irkin & Korukluoglu, 2007; Raut & Karuppaiyl, 2014)
	Onion	<i>Allium cepa</i>	Bulb	<i>S. aureus; S. Enteritidis; A. nige; Penicillium cyclopium; Fusarium oxysporum</i>	(Benkeblia, 2004)
6. Betulaceae	Birch tar	<i>Betula pendula</i>	Bud	<i>E. coli; S. aureus; B. cereus; Micrococcus luteus</i>	(Başer & Demirci, 2007)

(Continued)

Table 6.3 (Continued)

Plant family	Common name	Species	Part of plant	Target organisms	References
7. Cupressaceae	Cade	<i>Juniperus oxycedrus</i>	Wood	<i>Aspergillus niger</i> ;	(Sridhar <i>et al.</i> , 2003)
	Cedarwood	<i>Cupressus funebris</i> and <i>Juniperus sp</i>	Wood	<i>Alternaria alternate</i> ; <i>Aspergillus niger</i> ; <i>A. flaus</i> ; <i>A. fumigatus</i> ; <i>Candida</i> spp.;	(Mimica-Dukic <i>et al.</i> , 2004; Raut & Karuppayil, 2014)
	Cypress	<i>Cupressus sempervirens</i>	Leaf/twig		
	Juniper berry	<i>Juniperus communis</i>	Friut	<i>Candida</i> spp.;	(Pepeljnjak <i>et al.</i> , 2005)
8. Geraniaceae	Rose geranium	<i>Pelargonium graveolens</i>	Leaf	<i>A. hydrophila</i> ; <i>Alcaligenes faecalis</i> ; <i>B. subtilis</i> ; <i>E. coli</i> ; <i>E. aerogenes</i> ; <i>E. faecalis</i> ; <i>K. pneumonia</i> ; <i>Proteus vulgaris</i> ; <i>S. aureus</i> ; <i>Serratia marcescens</i> ; <i>Micrococcus luteus</i> ; <i>Moraxella</i> spp.; <i>Yersinia enterocolitica</i> ;	(Trlpath <i>et al.</i> , 2011; Speranza <i>et al.</i> , 2010; Lalli & Jacqueline, 2006; Dorman & Deans, 2000; Boukhatem <i>et al.</i> , 2013; Hsouna & Hamdi, 2012; Aggarwal <i>et al.</i> , 2000)
9. Juglandaceae	Common walnut	<i>Juglans regia</i>	Friut	<i>B. Subtilis</i> ; <i>E. coli</i> ; <i>Klebsiella pneumonia</i> ; <i>Proteus vulgaris</i> ; <i>Pseudomonas aeruginosa</i> ; <i>S. aureus</i> ; <i>Salmonella typhimurium</i> ;	(Pereira <i>et al.</i> , 2007; Dorman & Deans, 2000)
10. Lamiaceae	Basil	<i>Ocimum basilicum</i>	Herb	<i>S. aureus</i> ; <i>E. coli</i> , <i>B. subtilis</i> ; <i>Pasteurella multocida</i> and pathogenic fungi <i>A. niger</i> ; <i>Mucor mucedo</i> ; <i>Fusarium solani</i> ; <i>Botryodiplodia theobromae</i> ; <i>Rhizopus solani</i>	(Hussain <i>et al.</i> , 2008)
	Clary sage	<i>Salvia sclarea</i>	Flowering herb	<i>E. coli</i> ; <i>Klebsiella pneumonia</i> ; <i>S. aureus</i> ; <i>Salmonella typhimurium</i>	(Hammer <i>et al.</i> , 1999)

Lavender	<i>Lavandula angustifolia</i> , <i>Lavandula latifolia</i>	Leaf, flower	<i>S. aureus</i> ; <i>albicans</i> ; <i>Acinetobacter baumannii</i> ; <i>Klebsiella pneumoniae</i> ; <i>E. coli</i> ; <i>S. aureus</i>	(Lodhia <i>et al.</i> , 2009; Hammer <i>et al.</i> , 1999)
Marjoram	<i>Origanum majorana</i>	Herb	<i>Candida albicans</i> ; <i>E. faecalis</i> ; <i>E. coli</i> ; <i>S. aureus</i> ; <i>S. typhimurium</i> ; <i>Serratia marcescens</i> ; <i>Acinetobacter baumannii</i> ; <i>Aeromonas sobria</i>	(Hammer <i>et al.</i> , 1999)
Oregano	<i>Origanum</i> spp., <i>Thymbra spicata</i> , <i>Coridothymus capitatus</i> , <i>Satureja</i> spp., <i>Lippia Graveolens</i>	Herb	<i>Aeromonas hydrophila</i> ; <i>Alcaligenes faecalis</i> ; <i>Bacillus subtilis</i> ; <i>Enterobacter aerogenes</i> ; <i>E. cloacae</i> , <i>E. faecalis</i> , <i>Klebsiella pneumoniae</i> , <i>Pseudomonas aeruginosa</i> , <i>S. aureus</i> , <i>Serratia marcescens</i> , <i>Moraxella</i> spp.;	(Dorman & Deans, 2000; Lambert <i>et al.</i> , 2001; Nostro <i>et al.</i> , 2007; Chaudhry <i>et al.</i> , 2007)
Pennyroyal	<i>Mentha pulegium</i>	Herb	<i>S. aureus</i> ; <i>B. subtilis</i> ; <i>B. cereus</i>	(Mahboubi & Haghi, 2008)
Peppermint	<i>Mentha piperita</i>	Leaf	<i>E. coli</i> ; <i>Enterobacter aerogenes</i> ; <i>Klebsiella pneumoniae</i> ; <i>S. aureus</i> ; <i>Mucor ramannianus</i> ;	(Iskan <i>et al.</i> , 2002; Hajlaoui <i>et al.</i> , 2009; Raut & Karuppaiyl, 2014)
Rosemary	<i>Rosmarinus officinalis</i>	Leaf	<i>S. aureus</i> ; <i>E. coli</i> ; <i>B. Subtilis</i> ; <i>Alternaria alternate</i> ; <i>A. parasiticus</i> ; <i>A. fumigatus</i> ; <i>Botrytis</i> spp.; <i>Fusarium</i> spp.;	(Santoyo <i>et al.</i> , 2005; Sridhar <i>et al.</i> , 2003)
Sage	<i>Salvia</i> spp.	Herb	<i>E. coli</i> ; <i>Klebsiella pneumoniae</i> ; <i>Salmonella typhimurium</i> ;	(Hammer <i>et al.</i> , 1999; Şahin <i>et al.</i> , 2003)
Savoury	<i>Satureja hortensis</i>	Leaf	<i>A. flavus</i> ; <i>A. parasiticus</i> ; <i>Bacillus</i> spp.; <i>E. coli</i> ; <i>L. monocytogenes</i> ; <i>S. typhimurium</i> ; <i>S. aureus</i>	(Dikbas <i>et al.</i> , 2008; Oussalah <i>et al.</i> , 2007)
Spearmint	<i>Mentha spicata</i> , <i>Mentha gracilis</i>	Leaf	<i>E. coli</i> ; <i>Enterobacter aerogenes</i> ; <i>Klebsiella pneumoniae</i> ; <i>S. aureus</i> ;	(Hajlaoui <i>et al.</i> , 2009; Hammer <i>et al.</i> , 1999)
Thyme	<i>Thymus vulgaris</i>	Herb	<i>Salmonella enteritidis</i> , <i>A. Hydrophila</i> ; <i>Alcaligenes faecalis</i> ; <i>B. subtilis</i> ; <i>E. coli</i> ; <i>E. faecalis</i> ; <i>Klebsiella pneumoniae</i> ; <i>Proteus vulgaris</i> ; <i>Pseudomonas aeruginosa</i> ; <i>S. aureus</i> ; <i>Serratia marcescens</i> ; <i>Micrococcus luteus</i> ; <i>Moraxella</i> spp.; <i>Yersinia enterocolitica</i>	(Dorman & Deans, 2000; Bozin <i>et al.</i> , 2006; Rota <i>et al.</i> , 2004; Hammer <i>et al.</i> , 1999)

(Continued)

Table 6.3 (Continued)

Plant family	Common name	Species	Part of plant	Target organisms	References
	Lemon balm	<i>Melissa officinalis</i>	Leaf	<i>Pseudomonas aeruginosa</i> ; <i>E. coli</i> ; <i>Salmonella</i> <i>Sarcina lutea</i> ; <i>Micrococcus flavus</i> ; <i>S. aureus</i> ; <i>Bacillus subtilis</i> ; <i>Salmonella typhimurium</i> ;	(Mimica-Dukic <i>et al.</i> , 2004)
	Zataria	<i>Zataria multiflora</i> Boiss	Leaf	<i>S. aureus</i> ; <i>B. cereus</i> ; <i>E. coli</i> ; <i>S. typhi</i> ; <i>Proteus vulgaris</i> ; <i>Shigella flexneri</i> ; <i>Candida</i>	(Fazeli <i>et al.</i> , 2007; Mansour <i>et al.</i> , 2010, Mahmoudabadi <i>et al.</i> , 2007)
	Blue mint	<i>Ziziphora clinopodioides</i>		<i>B. subtilis</i> ; <i>E. coli</i> ; <i>Aspergillus fumigatus</i> and <i>Aspergillus flavus</i> ; <i>E. faecalis</i> ; <i>Klebsiella pneumonia</i> ; <i>Pseudomonas aeruginosa</i> ; <i>S. aureus</i> ;	(Khosravi <i>et al.</i> , 2011; Ozturk & Ercisli, 2007)
	Wild mint	<i>Mentha longifolia</i>	Leaf	<i>E. coli</i> ; <i>Enterobacter aerogenes</i> ; <i>Klebsiella pneumonia</i> ; <i>S. aureus</i> ;	(Hajlaoui <i>et al.</i> , 2009)
11. Lauraceae					
	Camphor	<i>Cinnamomum camphora</i>	Wood	<i>E. coli</i> ; <i>proteus. vulgaris</i> ; <i>S. aureus</i>	(Li <i>et al.</i> , 2006)
	Cinnamon	<i>Cinnamomum zeylanicum</i> , <i>Cinnamomum cassia</i> , <i>Cinnamomum zeylanicum</i>	Leaf, bark	<i>Y. lipolytica</i> ; <i>P. membranaefaciens</i> ; <i>P. roquefortii</i> ; <i>M. plumbe</i> ; <i>Eurotium</i> spp.; <i>D. hansenii</i> , <i>Z. rouxii</i>	(Matan <i>et al.</i> , 2006)
	Rosewood	<i>Aniba rosaedoura</i>	Wood	<i>Acinetobacter baumannii</i> ; <i>Aeromonas sobria</i> ; <i>Candida albicans</i> ; <i>E. faecalis</i> ; <i>E. coli</i> ; <i>Klebsiella pneumonia</i> ; <i>Salmonella typhimurium</i> ; <i>Serratia marcescens</i> ; <i>S. aureus</i>	(Hammer <i>et al.</i> , 1999)

12. Myrtaceae

Bay	<i>Pimenta racemosa</i>	Leaf	<i>Acinetobacter baumannii</i> ; <i>Aeromonas sobria</i> ; <i>Candida albicans</i> ; <i>E. faecalis</i> ; <i>E. coli</i> ; <i>Klebsiella pneumoniae</i> ; <i>Salmonella typhimurium</i> ; <i>Serratia marcescens</i> ; <i>S. aureus</i> ; <i>Pseudomonas aeruginosa</i>	(Hammer <i>et al.</i> , 1999)
Cajuput	<i>Melaleuca leucandendron</i>	Leaf	<i>Acinetobacter baumannii</i> ; <i>Aeromonas sobria</i> ; <i>Candida albicans</i> ; <i>E. faecalis</i> ; <i>E. coli</i> ; <i>Serratia marcescens</i> ; <i>S. aureus</i>	(Hammer <i>et al.</i> , 1999)
Clove	<i>fme</i>	Leaf/bud	<i>A. Hydrophila</i> ; <i>Alcaligenes faecalis</i> ; <i>B. subtilis</i> ; <i>E. coli</i> ; <i>Enterobacter aerogenes</i> ; <i>E. faecalis</i> ; <i>Haemophilus influenzae</i> ; <i>Klebsiella pneumoniae</i> ; <i>Proteus vulgaris</i> ; <i>Pseudomonas aeruginosa</i> ; <i>S. aureus</i> ; <i>Serratia marcescens</i> ; <i>Micrococcus luteus</i> ; <i>Moraxella spp.</i> ; <i>Yersinia enterocolitica</i> ; <i>Aspergillus niger</i> ; <i>A. fumigatus</i>	(Dorman & Deans, 2000; Rhayour <i>et al.</i> , 2003; Saeed & Tariq, 2008; Fabio <i>et al.</i> , 2007; Joshi <i>et al.</i> , 2011)
Eucalyptus	<i>Eucalyptus</i> spp.	Leaf	<i>S. aureus</i> ; <i>E. coli</i> ; <i>Candida albicans</i> ; <i>Haemophilus influenzae</i> ; <i>Klebsiella pneumoniae</i> ;	(Sartorelli <i>et al.</i> , 2007; Salari <i>et al.</i> , 2006)
Tea tree	<i>Melaleuca alternifolia</i>	Leaf	<i>Candida albicans</i> ; <i>Candida glabrata</i> ; <i>Saccharomyces cerevisiae</i> , <i>E. coli</i> ; <i>E. faecalis</i> ; <i>Mycobacterium avium</i> ; <i>Pseudomonas aeruginosa</i> ; <i>S. aureus</i> ;	(Cox <i>et al.</i> , 2000; Hammer <i>et al.</i> , 2004; Hammer <i>et al.</i> , 1999; Carson <i>et al.</i> , 2006)

13. Myristicaceae

Nutmeg	<i>Myristica fragrans</i>	Seed	<i>B. Subtilis</i> ; <i>E. Coli</i> ; <i>Klebsiella pneumoniae</i> ; <i>Proteus vulgaris</i> ; <i>S. aureus</i> ; <i>Serratia marcescens</i> ; <i>Micrococcus luteus</i> ; <i>Yersinia enterocolitica</i> ;	(Narasimhan & Dhake, 2006; Indu <i>et al.</i> , 2006; Dorman & Deans, 2000)
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Table 6.3 (Continued)

Plant family	Common name	Species	Part of plant	Target organisms	References
14. Poaceae	Citronella	<i>Cymbopogon Spp</i>	Leaf		
	Gingergrass	<i>Cymbopogon martinii</i>	Leaf	<i>Alternaria alternate</i> ; <i>Aspergillus niger</i> ; <i>A. Flavus</i> ; <i>Candida</i> spp.; <i>Penicillium</i> spp.; <i>Fusarium</i> spp.;	(Sridhar <i>et al.</i> , 2003; Raut & Karuppaiyl, 2014)
	Lemongrass	<i>Cymbopogon fl exuosus</i> , <i>Cymbopogon citratus</i>	Leaf	<i>Alternaria alternate</i> ; <i>Aspergillus niger</i> ; <i>Candida</i> spp.; <i>Penicillium</i> spp.; <i>Fusarium</i> spp.;	(Sridhar <i>et al.</i> , 2003; Raut & Karuppaiyl, 2014)
15. Pinaceae	Pine needle	<i>Pinus densiflora</i> <i>Pinus koraiensis</i>	Leaf/twig	<i>Salmonella typhimurium</i> , <i>L. monocytogenes</i> ; <i>E. coli</i> ; <i>Klebsiella pneumonia</i> ; <i>Salmonella typhimurium</i> ;	(Hong <i>et al.</i> , 2004; Bakkali <i>et al.</i> , 2008; Singh <i>et al.</i> , 2002)
16. Piperaceae	Pepper	<i>Piper nigrum</i>	Friut	<i>A. hydrophila</i> ; <i>Alcaligenes faecalis</i> ; <i>B. subtilis</i> ; <i>E. coli</i> ; <i>Enterobacter aerogenes</i> ; <i>E. faecalis</i> ; <i>Klebsiella pneumonia</i> ; <i>Pseudomonas aeruginosa</i> ; <i>S. aureus</i> ; <i>Serratia marcescens</i> ; <i>Micrococcus luteus</i> ;; <i>Moraxella</i> spp.; <i>Yersinia enterocolitica</i> ; <i>Candida</i> spp.;	(Dorman & Deans, 2000; Reddy <i>et al.</i> , 2004; Reshmi <i>et al.</i> , 2010; Karsha & Lakshmi, 2010; Raut & Karuppaiyl, 2014)
17. Rutaceae	Amyris	<i>Amyris balsamifera</i>	Wood		
	Bergamot	<i>Citrus aurantium</i> ;	Fruit peel	<i>Kluyveromyces fragilis</i> , <i>Rhodotorula rubra</i> , <i>Candida albicans</i> , <i>Hanseniaspora guilliermondii</i> and <i>Debaryomyces hansenii</i> ;	(Kirbaşlar <i>et al.</i> , 2009)
	Grapefruit	<i>Citrus limon</i> ;		<i>S. aureus</i> ; <i>B. cereus</i> ; <i>Mycobacterium smegmatis</i> ;	
	Lemon	<i>Citrus sinensis</i>		<i>L. monocytogenes</i> ; <i>Micrococcus luteus</i> ; <i>E. coli</i> ;	
	Mandarin			<i>Klebsiella pneumonia</i> ; <i>Pseudomonas aeruginosa</i> ; <i>Proteus vulgaris</i>	
	Orange				

18. Rosaceae	Rose	<i>Rosa damascena</i>	Flower	<i>Xanthomonas axonopodis</i> spp.	(Basim & Basim, 2003)
19. Santalaceae	Sandalwood	<i>Santalum album</i>	Wood	<i>Candida albicans</i> ; <i>E. faecalis</i> ; <i>S. aureus</i>	(Hammer <i>et al.</i> , 1999)
20. Styracaceae	<i>Tamarix boveana</i>	Tamarix boveana	Flower/leaf/ stem	<i>S. aureus</i> <i>S. epidermidis</i> <i>E. coli</i> <i>Pseudomonas aeruginosa</i> <i>Micrococcus luteus</i> <i>Salmonella typhimurium</i> <i>Fusarium oxysporum</i> <i>Aspergillus niger</i> <i>Penicillium</i> spp.; <i>Alternaria</i> spp.	(Saidana <i>et al.</i> , 2008)
21. Zingiberaceae	Cardamom	<i>Elettaria cardamomum</i>	Seed	<i>S. aureus</i> ; <i>B. cereus</i> ; <i>E. coli</i> ; <i>S. typhi</i>	(Singh <i>et al.</i> , 2008)

6.3 Synergistic and Antagonism Effect of Essential Oils with Other Antimicrobials

As mentioned before, the antimicrobial activity of essential oils are often related to their major components, even though the presence of minor compounds and the ratio between active constituents also play crucial role due to synergistic effect. Generally, antimicrobial potency of essential oils can be improved in combination of other antimicrobial agents or preservation methods. Furthermore, organoleptic impact of essential oils would be reduced by their simultaneous application with other antimicrobials in food products. Synergism means 'to work together' and is defined as the greater effect of the combined antimicrobials is observed compared to the sum of the individual effects.

However, it should be noted that using a mixture of antimicrobials might have no enhancing influence or even reduce inhibitory action of essential oils depending on antimicrobial type and environmental conditions.

When combined effect of antimicrobials is equal to the sum of the individual effects, it is known as additive effect, and while the mixture of antimicrobials has less inhibitory effect than when individually used, antagonism is observed (Burt, 2004).

Interaction effect between antimicrobials is often expressed by fractional inhibitory concentration (FIC) index which is calculated based on minimum inhibitory concentration (MIC) of antimicrobial agents according to following equations;

$$FIC_A = MIC_{A+B} / MIC_A, FIC_B = MIC_{B+A} / MIC_B \text{ and,} \\ FIC_{Index} = FIC_A + FIC_B,$$

where A = antimicrobial A and B = antimicrobial B. To interpret FIC_{Index} value, theoretically, 1 is considered as a cut-off valve and FIC close to 1 implies additive impact, while below and above 1 indicate synergistic and antagonistic effects, respectively. However, in most references, a more common system is used as follow; $FIC \leq .05$, synergy; $FIC = 0.5-4$, no interaction (additive); $FIC \geq 4$, antagonism (Hyldgaard *et al.*, 2012; Sánchez-González *et al.*, 2011).

Application of single essential oils as food preservative may be restricted due to high concentration required to reach a guaranteed antimicrobial inhibition and ensure food safety which lead to undesirable organoleptic characteristics such as becoming off-flavour. Synergetic effect of essential oils application together or in combination with other antimicrobial compounds could act as an additional hurdle, resulting in lower concentration of essential oils required to antimicrobial activities, which retain food sensorial acceptability and safety. Moreover, essential oils may use along with different food preservation methods such as refrigeration, modified atmosphere packaging, high pressure, pulsed electric field and so on, in order to retard growth of pathogenic and spoilage-forming microorganisms (Pesavento *et al.*, 2015).

Literature survey has demonstrated that whole oil showed better antibacterial efficacy than only a combination of major volatiles of the oil (Bajpai *et al.*, 2012).

6.4 Interaction Between Essential Oils and Essential Oils with Other Food Antimicrobials

Previous studies have demonstrated that antimicrobial efficacy of combination of two or more essential oils or one oil with another preservative could increase compared to single essential oil (Bajpai *et al.*, 2012). For example, synergism between cinnamaldehyde and carvacrol has been reported when used for inactivation of some major food-borne pathogens including *E. coli*, *S. aureus* and *S. enteritidis* (Ye *et al.*, 2013).

Mixture of oregano and thyme essential oils in ratio of 1 to 4, resulted in high antibacterial efficiency on *E. coli* than when used individually. However, oregano in combination with rosemary has been shown to exert additive effect (Gutierrez *et al.*, 2008).

Other antimicrobials than essential oils have been reported to have interaction with essential oils to outcome synergistic, additive or antagonistic effects. Amongst them salts and bioactive peptides such as bacteriocins have drawn considerable interest.

The combined application of essential oils and bacteriocins especially nisin can work as additive or even antagonist, depending on bacteria and antimicrobials type and different growth conditions. It has been shown that antibacterial activity of a mixture of nisin and carvacrol increased at low pH and low temperature against *B. cereus*. However, an additive effect was observed using combination of nisin and essential oil of *thymus vulgaris* at neutral pH (Turgis *et al.*, 2012). This indicated that inhibitory potency of both nisin and carvacrol activities improve at low pH and low temperature. On the other hand, a combination of nisin and carvacrol did not enhance antimicrobial activity of them individually against *salmonella typhimurium* (additive effect) (Lee & Jin, 2008).

Mechanism of essential oils and different antimicrobial agent's interaction inducing synergy is not completely understood. Nevertheless, it is postulated that each antimicrobial could possess individual and distinct mode of action, which may vary from one to another. However, main pathways proposing for description synergistic effect of antimicrobial compounds, include; (1) interaction one antimicrobial with cell membrane or/and wall which may facilitate penetration of other antimicrobials or may improve formation of membrane pore in cells, (2) more than one antimicrobial may interact with cell membrane or/and wall which may degrade the cell integrity and induce leakage of critical cell compounds which in turn change pH and electrical gradient and the proton motive force and (3) acting as sequential barriers for different steps of a specific biochemical route. Although, there is no clear knowledge about antagonistic impact of antimicrobials, it has been suggested that it takes place when the antimicrobials interact together and/or when they adversely compete with each other for a target site on microorganism cells (Hyldgaard *et al.*, 2012).

6.5 Food Packaging Containing Essential Oils

6.5.1 Antimicrobial Activity of Packaging Containing Essential Oils

Inhibitory action of essential oils against food microorganisms, turn them as promising antimicrobial agents for food application. However, direct incorporation of essential oils to food matrices impose some restrictions due to interaction with food components,

which could reduce their inhibitory potency, possible lower distribution rate to target sites and strong flavour and odour, which may adversely affect on organoleptic properties of food (Campos *et al.*, 2011). One way to minimise these limitations is to entrap essential oils in capsules or packaging matrix. A product's packaging is a crucial part of food chain processing which act as an extra barrier to protect food from environmental damages and cross-contamination. Incorporation of essential oil to food packaging films turns them to active form of packaging which not only serve their primary functions as a pack, but also act as a tool to carry essential oil for improvement safety and shelf-life of packed food. Essential oil in film matrix could contact directly with food surface or indirectly through head space. In both cases, interaction of essential oil with food ingredients and also concern about undesirable organoleptic impact especially for indirect contact would be lessened, providing superior usage of essential oils. Moreover, it is worthy to note that in most food products, microorganisms are mainly located on the food surface, which would be surrounded with film containing essential oil (Han, 2005).

Generally, there are two main categories for antimicrobial packaging systems, which include packages with immobilised antimicrobial agent on their surface by ion or covalent linkages in which antimicrobials cannot release, then straight contact of food and antimicrobial film is required to antimicrobial efficiency and those which in antimicrobials incorporated in their bulk mass or applied on their surface, which are able to migrate onto food surface or into package head space (López, 2007).

Different kind of polymers can be used to prepare antimicrobial films based on essential oils; synthetic (petrochemical) polymers such as polyethylene, polypropylene, polystyrene and polyvinyl chloride and biopolymers such as carbohydrates, proteins and lipids.

6.5.1.1 Biopolymers

Carbohydrates and proteins possess good film-forming ability with excellent oxygen barrier property, often transparent appearance and environmentally friendly behaviour. However, because of their poor mechanical and water vapor resistance performance, they often considered as additional barrier/stress factor for food preservation and thus are still preferentially used in combination to conventional packaging.

Most of biopolymers used in film matrices are hydrophilic in nature, then form water-soluble film structure which is in opposite of hydrophobic character of essential oils. For this reason, biopolymer based-films containing essential oils are regarded as emulsified films, then it is necessary to emulsify oil phase (essential oil) in aqueous phase (biopolymer solution containing plasticiser) before casting for film formation. Similar to other emulsions, to stabilise the obtained film-forming dispersions emulsifier(s) is required.

Therefore, application of high-energy techniques is a necessary step to prepare emulsified films which is usually done first by a high-speed mixer for preparation of coarse emulsion and then by a homogeniser to make enough small droplets. Homogenisation helps uniformly distribute of essential oil droplets in dried film matrix, which is important for film to have steady inhibitory effect on food product. Moreover, it has been shown that reduction size of essential oil droplets in different ways such as using microfluidiser could increase considerably antimicrobial activity, which can be attributed to increased surface area of droplets (Otoni, 2014).

Tweens, especially 80, are the most common emulsifier using in antimicrobial emulsified films, which is shown that decrease inhibitory action of essential oils in *in vitro* due

to neutralise of phenolic compounds of essential oils. However, because of little amount of tween (usually 0.1 to 0.5% v/v based on essential oil concentration) required to emulsify the film-forming dispersion, and immediate drying the film-forming dispersion, it does not seem tweens cause reduction in antimicrobial activity of dried films. Martínez-Abad *et al.* reported that the addition of tween 20 and 80 into polycaprolactone film incorporated with cinnamaldehyde, at concentration of 0.1% and 0.5%, did not have significant effect on antimicrobial activities of film against *Salmonella enterica* and *L. monocytogenes* (Martínez-Abad *et al.*, 2013).

Plasticisers such as polyethylene glycol, sugar-alcohol and especially glycerol are generally used to improve flexibility of polymeric films and thus prevention of brittleness. It does not seem that these compounds have positive or negative effect on antimicrobial action of essential oils.

The emulsified biopolymer-based film are usually produced by casting methods, in which film-forming dispersion is uniformly distributed onto a surface and allowed to dried under controlled condition. Drying temperature often keep low because high temperature may cause non-cohesive structure. Essential oil are volatile and relatively heat reliable but it is not expected these low temperatures evaporate or/and degrade essential oils. Furthermore, most carbohydrate and protein solutions start forming a network structure during the first of drying process, which can entrap the essential oil droplets and minimise their evaporation and then loss of antimicrobial activity. On the other hand, adjust a desired antimicrobial final concentration during formulation can be compensated evaporation of volatile essential oils in dried films (Du *et al.*, 2011).

6.5.1.2 Synthetic Polymers

In spite of rising concern over the use of synthetic polymers due to their low biodegradability, they are still common materials for food packaging because they have some advantages, including good mechanical and vapor resistance properties, transparency, easy heat sealability and low cost. To prepare the antimicrobial plastic films again migrating and non-migrating packaging can be used. In more common migrating systems, essential oils are incorporated to film solution or molten followed by solvent casting technique and heat processing methods such as extrusion for film formation.

In converse to biopolymer-based films, distribution of essential oils in plastic films can be controlled much more simple because of hydrophobic nature of the most petroleum driving polymer and their compatibility with essential oils. However, high temperature and pressure required during melting processing such as extrusion method may cause some vitalisation and deformation of active compounds and lead to reduce antimicrobial activity (Sung *et al.*, 2014; Du *et al.*, 2011; Kuorwel *et al.*, 2011). Therefore, avoidance of harsh conditions, in particular, high temperature during processing of plastic films incorporated with essential oil should be considered (Quintavalla & Vicini, 2002).

Nevertheless, it is worthy to consider that thermal processing causing changes in molecular structure of essential oil constituents may convert them to antimicrobials with higher inhibitory action on food microorganisms. Sung *et al.* (2011) incorporated *Allium sativum* essence oil into low density polyethylene/ethylene vinyl acetate (LDPE/EVA) co-polymer film which was prepared by extrusion technique at 170°. Allicin as a main antimicrobial component of *Allium sativum* essence oil is heat liable and it was expected that no allicin remained at obtained film. However, the antimicrobial films

had inhibitory effect on beef-related pathogenic and spoilage bacteria which attributed to antimicrobial activity of ajoenes formation from allicine degradation during extrusion (Solano & de Rojas Gante, 2012). This result implies importance of having information about compositional changes of essential oil during heating or high pressure, which is not studied enough yet.

Ionising method is a way to form to immobilise essential oils on the surface of plastic films and formation of a non-migrating polymeric packaging. In this technique, the surface of polymeric film is ionised by radiation or corona discharge to make more reactive of molecules of polymer surface to better deposition of essential oils on film face. On the other hand, although ionising treatment can apply at lower temperature with lower damages to antimicrobial activity of essential oils, Solano *et al.* showed that LDPE films containing oregano oil developed by extrusion had greater inhibition on *L. monocytogenes*, *Salmonella thyphimurium* and *Escherchia coli O157:H7* compared to ionising method (corona discharge) who suggested that antimicrobial inhibitory action of ionising method might be improved by varying time, power and electrode type (Solano & de Rojas Gante, 2012).

A novel strategy to reduce problems due to application of each synthetic or biopolymer-based films individually may be is to combine these polymer films by laminate technique. In these methods essential oil is incorporated into biopolymer film layer at low temperature, which is attached to synthetic film layer treated by different kinds of plasma to form a composite film. A plasma is a fully or partially ionised gas containing positive and negative ions and radicals (Pankaj *et al.*, 2014), which modify the surface of synthetic polymeric film by formation of polar groups on its surface, thus improved its surface hydrophilicity and its adhesion to biopolymer layer.

Thus, biopolymer-based film serves as a carrier for heat-labile essential oils in addition to its natural origin making it more safe to even directly contact with food surface.

On the other hand, synthetic film attached to natural polymer layer, which is an outside layer of packaging, produce a good mechanical and water vapor barrier. Furthermore, outer polymeric layer serves as a hurdle to protect essential oils against evaporation and loss during storage of packed food lead to retain its antimicrobial efficiency.

Finally, using synthetic and biopolymers in combination, for production of packaging containing of essential oils compensate each other, thus likely leading to a composite layered film with improved functionality such as antimicrobial activity.

6.5.2 Antimicrobial Activity of Packaging in Vapor Phase

Most of antimicrobial agents used in active films are non-volatile compounds with no ability to release to head space surrounding the packed food; therefore, it is necessary to bring the active films upon direct contact with food surface where microorganisms are mainly located. However, essential oils often have a relatively high vapor pressure and there is some evidence that essential oils in vapor phase have greater inhibitory effect against both bacteria and fungi, which means that lower amount of essential oils is needed to retard or reduce growth of foodborne microorganisms compared to liquid phase (direct contact). Hydrophobic molecules has been suggested that form micelles in aqueous phase and thus reducing affinity of lipophilic components of essential oils to attach with microorganism cell, whereas presence of essential oils in gaseous form cause their free movement of molecules resulting in facilitating diffusion into cell

membrane (Martínez-Abad *et al.*, 2013). In addition, Tyagi and Malik (2011) reported higher monoterpene hydrocarbon constituents in vapor of *menta piperita* oil compared to oxygenated monoterpenes that inflicted serious damages to microbial cells (Tyagi & Malik, 2011).

Effectiveness of antimicrobial activity of essential oils in gaseous phase without requiring straight contact with microorganisms can be considered as a promising way to develop antimicrobial packaging.

Volatility of essential oils provides this possibility to vapor phase transfer from film into interior atmosphere in the packaging. The use of essential oil in vapor phase may offer potential advantages such as creating efficient and protective antimicrobial atmosphere with no necessity for direct touch, resulting in slight organoleptic impact inducing by change in natural taste and odour of the food by being more than the acceptable essential oil flavour limit.

6.5.3 Release Properties of Essential Oils From Packaging

Efficiency of antimicrobial films depends on essential oils-releasing properties, which in turn influenced by some parameters such as type of polymers, interactions between the antimicrobial and the polymer chains, water content of packed food and environmental conditions such as relative humidity and temperature (Avila-Sosa *et al.*, 2012, Ponce *et al.*, 2008).

The used polymer to form the film, primarily determines the characteristics of the film matrix and also its release and thus antimicrobial properties depending on its nature and interaction capability with essential oil constituents. The films produced by polymers with inherent antimicrobial activity such as chitosan show synergistic effect when added with essential oils. This is probably due to the fact that chitosan in solid form, in contrast with entrapped essential oils, only inhibit growth of microorganisms which are located close to the active site of polymer (Sánchez-González *et al.*, 2011). In films incorporated with essential oils, the oil droplets are physically and/or chemically entrapped into polymer matrix. The presence of chemical bonds between essential oil and functional groups of polymer causes strong interaction that reduce migration rate of active components, which allowed slower diffusion of essential oil into packaging-food contact and thus gradual release on packed food surface. In some application, slow release of bioactive compounds from packaging system is necessary to improve shelf-life of products during storage. Formation of covalent bonds between amino groups with of chitosan and functional group of cinnamaldehyde of cinnamon oil led to films with improved mechanical, water vapor permeability and antibacterial properties. However, if there is enough strong the cross-linking of polymer and essential oil, it would be retarded migration of oil molecules and exertion of antimicrobial activity, for example, chemical bonds amino groups of casein film and carboxyl groups of oleoresins (Ponce *et al.*, 2008).

When inert polymers are used for film formation, there would be no significant molecular interaction with essential oils, hence retaining of active compounds would be less impressively and their diffusion can occur rapidly across film. Existing very weak or no polymer-essential oil interaction due to incompatibility between them may result in rapid liberation (burst release) of antimicrobial agents into food which can decrease shelf-life of packed food due to inactivation of most essential oils components during early periods of storage.

Contrary to synthetic films, most biopolymer-based films have hydrophilic nature and may be absorbed water and fractured upon direct exposure to wet food surface during storage and again causing a burst release which is often undesirable for food preservation. Application of edible films containing essential oils vapor phase with distance from moist food surface, protect hydrophilic film against swelling, destruction and thus burst release.

Environmental changes such as relative humidity and temperature also influence on essential oils diffusion through films. Generally, storage of food packed at high temperature can accelerate diffusion of essential oils across synthetic or biopolymer-based film, whereas low temperature decrease release rate. This can be attributed to increase evaporation and migration rate as temperature rise up, which may be regarded as a targeted antimicrobial release, since essential oil diffusion would be controlled by temperature and activity of antimicrobial packaging can be improved at abuse temperatures (Martínez-Abad *et al.*, 2013; Manso *et al.*, 2015).

However, relative humidity variation has been reported that did not affect on antimicrobial activity of polycaprolactone films containing cinnamaldehyde and allyl isothiocyanate at constant temperature. Whereas higher dependence of antimicrobial effectiveness on relative humidity has been observed on biopolymer-based films because of their hydrophilic nature (Martínez-Abad *et al.*, 2013).

6.6 Encapsulation of Essential Oils

Essential oils are valuable ingredients and have many applications in food and other industries. However, generally these oils are volatile and unstable compounds that can be easily evaporated and decomposed due environmental conditions such as light, oxygen, or temperature. These chemical and physical changes can be effectively minimised by an adequate formulation of essential oils. Encapsulation is one strategy to overcome these problems. Microencapsulation technique can be applied in the food industry for different reasons, including protection of the core substance from the external medium, reduction of the core materials evaporation or transfer rate to the outside medium, improvement of the handling by modification of physical characteristic of the original compounds, controlling the release of the core materials, masking unwanted flavour or taste of the core substances, uniform dispersion of the core materials in the food matrixes and lower local concentration and thereby reduce toxicity, reducing the agglomeration of the superfine powders and avoiding the reaction of the core materials to other components (Fang & Bhandari, 2010; Singh *et al.*, 2010; Desai & Jin Park, 2005). Many studies have been used polymeric matrixes to encapsulate essential oils. There are several approaches for producing polymeric particles loaded with essential oils such as spray drying, coacervation, expansion of supercritical solutions, inclusion and nanoprecipitation. The process of spray drying is the most widely technique used for encapsulation due to its flexibility and economic advantages and possibility of particles producing in continuous operation with good quality. Gum arabic, whey protein, inulin, different type of starch and maltodextrin are the common matrixes in spray drying process of essential oils. These biopolymers can also be used with each other. Amongst these, gum arabic is considered to be an excellent matrix because of its high solubility, low viscosity and good emulsifying properties, but due to some problems such as its

high cost and limited supply, commonly it is used with other biopolymers specially maltodextrin. For example, in *Lippia sidoides*, essential oils has been encapsulated in maltodextrin and gum arabic. In this study, the best encapsulation efficiency was reported in maltodextrin:gum arabic ratio of 0:1 (m/m) and wall materials:essential oil ratio of 4:1 (m/m). Spray-dried microparticles of *Lippia sidoides* also acted as antifungal agents (Fernandes *et al.*, 2008). Encapsulating matrix type could effect on retention, delivery and thereby antimicrobial activity of essential oils. Beirão da Costa *et al.* (2012) have been investigated the encapsulation of oregano essential oils in rice starch porous spheres, inulin and gelatine/sucrose by spray drying. Rice starch microparticles containing oregano showed the higher diffusion coefficients followed by gelatine/sucrose and inulin microcapsules. The most antimicrobial activity was found for oregano encapsulated in gelatine/sucrose microcapsules, while inulin and rice starch microparticles showed higher stability (Beirão da Costa *et al.*, 2012). Antimicrobial activity of essential oils could be retained or improved by encapsulation. Improvement of antimicrobial activity following application of spray-drying method for encapsulation was also reported. Lavandin essential oils that has been encapsulated by soy lecithin as carrier materials had higher antimicrobial activity against *E. coli* than to free form of essential oils. Encapsulated Lavandin showed 73% bacteria growth inhibition, while free essential oils had 45% growth inhibition against *E. coli* (Varona *et al.*, 2013). Particles containing essential oils could be produced in nano size. As nano-sized particles have more surface area to volume ratio, it is expected more solubility, higher bioavailability and improvement control release of Eosin nanoparticles. Hydrophobic natures of essential oils make them a good candidate for encapsulation in nanoparticles produced by nanoprecipitation. Carvacrol is an important component of essential oils has been encapsulated in poly(dl-lactide-co-glycolide (PLGA) nanocapsules by nanoprecipitation. Effects of this antimicrobial nanoparticle on the viscoelastic features of *S. epidermidis* biofilms have been investigated. Treatment of staphylococcal biofilms with nanocapsules containing carvacrol produced a markedly reduction in the elasticity and mechanical stability of biofilms that could enable the penetration of carvacrol into the deep core of staphylococcal biofilms (Iannitelli *et al.*, 2011). Another technique for production of polymeric particles containing essential oils is coacervation. This method has many benefits such as using of a simple technology, high encapsulation efficiency (over 90%) and not using of organic solvents. One example of encapsulation of essential oils via coacervation is *Pimenta dioica* that has been encapsulated by chitosan/k-carrageenan complex coacervation approach. Microencapsulated *P. dioica* essential oil showed antimicrobial efficacy against *Candida utilis*, *Bacillus cereus* and *Bacillus subtilis* (Dima *et al.*, 2014).

The use of emulsions and multiple emulsions as templates for producing essential oils loaded in microparticles is interesting because of their potential use with sensitive components. In a recent study, a multiple oil-in-water-in-oil (o/w/o) emulsion and ionic gelation technique was used to produce *Satureja hortensis* (savory) essential oil-loaded alginate microparticles. Alginate microparticles containing 3% v/v savory showed antimicrobial activity against *S. aureus*, *B. cereus*, *E. coli*, *S. typhimurium* and *P. aeruginosa*. The highest antimicrobial activity was found for *S. aureus*, with an inhibition-zone of about 304 mm². On the other hand, *P. aeruginosa* exhibited the highest resistance (Hosseini *et al.*, 2013).

Liposome is another one of the promising encapsulating material that can be used to increase the antimicrobial efficacy of the essential oils. There are a number of methods

for producing liposomes such as thin film hydration, reverse phase evaporation and supercritical fluid technology. Some of liposome-loaded essential oils or their components that exhibited antimicrobial activity are rose, lavender, *Artemisia afra*, *Eucalyptus globulus*, *Melaleuca alternifolia*, tea tree oil, *Zanthoxylum tingoassuiba*, p-cymene, geraniol, g-terpinene, thymol (Van Vuuren *et al.*, 2010; Varona *et al.*, 2011; Low *et al.*, 2013; Coimbra *et al.*, 2011; Detoni *et al.*, 2012; Cristani *et al.*, 2007). In this regard, tea tree oil that encapsulated in liposome by reverse phase evaporation method had extended release and enhancement in its antimicrobial efficacy (Low *et al.*, 2013).

6.7 Application of Essential Oils as Antimicrobial Agents in Different Food Products

6.7.1 Bakery Products

Fungi are the most prevalent microorganisms of bakery products spoilage that are responsible of serious economic problem due to formation of bad appearance, off-flavour and possibility of allergenic compounds and mycotoxins production (Llana-Ruis-Cabello *et al.*, 2015). Different moulds such as aspergillus, penicillium, cladosporium, neurospora and mucorales are the most common bakery products spoilage microorganisms due to a_w (0.75–0.90) of bakery products (Gutierrez *et al.*, 2008).

The most common methods of bread preservation such as freezing (increase in energy consumption), refrigeration (losses its freshness and increases staling) and addition of antimicrobial agent or salts have undesirable outcomes. But using synthetic preservatives such as sorbate, propionate, acetate and ethanol (Gutierrez *et al.*, 2008) is not coincident with consumer's demand for preservative free food (Pesavento *et al.*, 2015). So, essential oil is good substitution for these common bakery preservatives.

Nielsen and Rios (2000) compared the effect of modified atmosphere packaging (MAP) and active packaging containing mustard essential oil on the most important spoilage fungi of bread. *Penicillium commune*, *P. roqueforti*, *Aspergillus flavus* and *Endomyces fibuliger* grew at oxygen levels above 0.03% when the chalk mould *E. fibuliger* was able to grow in spite of oxygen absorbers. Application of high levels of CO₂ retarded fungal growth partially but not completely. They also applied 1, 10 or 100 ml spices and herbs volatile essential oils (EO) in filter paper placed in the lid of a petri dish inoculated with one of the commonly bread spoilage fungi. Mustard essential oil had the stronger effect than cinnamon, garlic and clove, while vanilla showed no inhibitory effect against the tested microorganisms. *P. roqueforti* and *A. flavus* was the most sensitive and most resistant microorganism, respectively. The chemical composition of mustard essential oil was investigated. Minimal inhibitory concentration (MIC) of allyl isothiocyanate as the main effective component ranged from 1.8 to 3.5 mg/ml in gas phase. The fungistatic or fungicidal effect of allyl isothiocyanate depended on the concentration of spores and effective component content. When the gas phase contained at least 3.5 mg/ml, AITC was fungicidal to all tested fungi (Turgis *et al.*, 2012).

Khaki, Sahari and Barzegar evaluated effect of three levels (0.05%, 0.1%, 0.15%) of essential oils of chamomile as antimicrobial compound in cake preparation during

75 days of storage. Their results showed existence of 0.15% chamomile in the sample led to better antimicrobial activity than control samples, but its activity was low in comparison to synthetic ones. However, this sample did not get high score in flavour, taste and overall acceptability (Lee & Jin, 2008).

In another study, antimicrobial effects of cinnamon essential oil on kolompe (a traditional cookie in Kerman-Iran) shelf-life was evaluated by Noorolahi *et al.* (2012). They compared yeast, mold, total count, *Enterobacteriaceae*, *E. coli*-positive *Staphylococci coagulase* and *Bacillus cereus* of sample containing different concentrations of cinnamon essential oil (0.05, 0.1, 0.15%) with the control samples. Aerobic microorganism, yeast and mold did not grow on the sample in 30th day (Ojagh *et al.*, 2010).

Ibrahium, El-Ghany and Ammar (2013) studied antimicrobial activity of clove essential oil on preservation of cake by determining the counts of yeasts, molds, coliforms and total bacteria during 28 days. All of tested microorganisms had less growth than control that was decreased with increasing of clove essential oil levels. In addition, sensory results showed acceptability of all cake samples except the sample containing 800 ppm clove essential oil (Appendini & Hotchkiss, 2002).

The effect of coriander (*Coriandrum sativum L.*) essential oil on cake shelf-life during 60-day storage at room temperature was studied by Darughe, Barzegar and Sahari (2012). They identified α -pinene (6.37%), limonene (7.17%), cyclo hexanol acetate (cis-2-tert.butyl-) (14.45%) and camphor (44.99%) as the main components in coriander essential oil with GC-MS. No fungi were grown at 0.15% of coriander essential in the cake (Martínez-Abad *et al.*, 2013).

Suhr and Nielsen (2003) studied antifungal effect of essential oils of clove, thyme, bay, lemon grass, cinnamon leaf, mustard, sage, orange and two rosemary oils by addition of 100 and 250 μL^{-1} essential oil to rye bread-based agar medium and addition of 136 and 272 μL^{-1} volatile oil in air around the real rye bread. In rye bread-based agar medium, clove and cinnamon showed the best inhibitory effect after thyme oil that was lower for sage and rosemary oils. In the second method in which oils was used as volatile in the air around the bread, mustard and lemon grass had maximum inhibitory effect, but orange, sage and one rosemary showed less effects (Du *et al.*, 2011).

In addition, other results presented the effect of method of essential oil addition on antifungal effects. In direct methods, larger phenolic compounds such as eugenol and thymol (thyme, cinnamon and clove) had maximum effect, whereas in volatiles method, smaller compounds such as allyl isothiocyanate and citral (mustard and lemongrass) had best effect (Sung *et al.*, 2014).

Application of antimicrobials seems not to be enough for long-term protection because of possible contamination of bread surface during handling. In addition, low sensory acceptability of high dosage of essential oils is another important point that limited direct using of them in food products. Therefore, addition of these natural antimicrobials to packages seems to suitable method to overcome these disadvantages of essential oils (Pesavento *et al.*, 2015).

Gutierrez *et al.* (2009) evaluated the effectiveness of using cinnamon essential oils in polypropylene based package on shelf-life of packaged bakery product. Finally, they could increase shelf-life of bakery products from 3 to 10 days with maximum sensory aspects and safety (Llana-Ruis-Cabello *et al.*, 2015).

6.7.2 Dairy Products

The high mortality rate of human listeriosis has attracted much attention on *L. monocytogenes* and possible infected food product in particular dairy products (Lee *et al.*, 2008). Salmonella is another infected bacteria that found in spoiled cheese (Lee *et al.*, 2008). In addition, mold contamination of different dairy products such as cheese during ripening and during consumer storing in refrigerator is a big problem for these manufacturers. Many species of aspergillus and penicillium are common fungal contaminants of cheese. The ancient solution for this problem is physically impregnating the product with spices, herbs or their oils (Quintavalla & Vicini, 2002; Kuorwel *et al.*, 2011).

The eugenol and thymol had inhibitory effect on the growth of *Penicillium citrinum* — NRRL 2274 and NRRL 2269 and production of citrinin in different Spanish cheeses named Cebreiro, Arzua-Ulloa and San Simon. The fungi growth rate was determined using colony diameters and the formation of citrinin was assessed by a rapid semi-quantitative fluorometric method that confirmed by RP-HPLC. 200 mgr/ml of eugenol could fully inhibited fungal growth in Arzua-Ulloa cheese, while it had no effect in Cebreiro cheese. Addition of 100 mgr/ml eugenol delayed citrinin production until the sixth day, while no citrinin was detected at a concentration of 150 mgr/ml of eugenol in Arzua-Ulloa cheese. Moreover, no citrinin was detected after five days in the case of thymol at the same concentration. Neither thymol nor eugenol could prevent the production of citrinin at the applied concentrations in Cebreiro cheese. These varies results may be due to diverse composition of different cheese (Kuorwel *et al.*, 2011).

Another study was carried out on effect of 0.1, 0.5 and 1% four plant essential oils including bay, clove, cinnamon against *L. monocytogenes* and *Salmonella enteritidis* at 4°C and 10°C in low-fat and full-fat soft cheese. They found important role of cheese composition on efficiency of the plant essential oils. One percent of all four oils could reduce *L. monocytogenes* to $<1.0 \log_{10} \text{cfu ml}^{-1}$ in the low-fat cheese, but an increase in fat content of cheese (full fat cheese), decreased the antilisteric effect of essential oil. In full-fat cheese, only clove oil could reduce *L. monocytogenes*. They found similar results about *S. enteritidis* (Tihminlioglu *et al.*, 2010). Different justification exists for this phenomenon. Fat can form a protective coat around bacteria that can protect them from foreign agents. In addition, fat can absorb the antibacterial agent and decrease their concentration in the aqueous phase that leads to decrease in antimicrobial effect. On the other hand, reduction in water content of food can reduce movement of antimicrobial agents. The protein can form complex with phenols and then decrease their bactericidal effects (Benkeblia, 2004; Tihminlioglu *et al.*, 2010). Higher acidity of food increased antimicrobial activity of essential oils as well, because of increase in hydrophobicity of essential oils constituents that help to better dissolving in the lipid part of the bacterial membrane. Furthermore, the higher salt content of food increase the antimicrobial effect of essential oils due to synergistic effect (Tassou *et al.*, 1995).

In another study the bactericidal activity of a mixture of essential oils (DMC) against *Enterobacter cloacae*, *L. monocytogenes*, *E. coli* 0157:H7, *S. aureus*, *Listeria innocua*, *Salmonella cholerasuis*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, *Micrococcus luteus* and *Lactobacillus brevis* were tested in culture media and Spanish soft cheese. The results showed all gram-positive bacteria were more sensitive (40 ppm) than gram-negatives and no antimicrobial effect was observed against

Ps. fluorescens in Spanish soft cheese. Addition of higher quantity (2500) of DMC had antimicrobial activity on *L. monocytogenes* but was not effective on *E. coli* O157:H7 (Szumny *et al.*, 2010).

6.7.3 Meat Products

Gram-negative psychrotrophic bacteria, mainly *Pseudomonas*, *Enterobacteriaceae* and also lactic acid bacteria are common microorganisms responsible for meat spoilage. Moreover, *L. monocytogenes*, *Salmonella typhimurium*, *Salmonella enteritidis*, *E. coli* O157:H7 and *Yersinia enterocolitica* are the most important reason of meat borne illnesses and deaths. In addition, microbial growth can lead to undesirable organoleptic properties during meat storage. Some of the traditional food preserving methods such as thermal processing, freezing, drying and irradiation cannot be used for some food products such as fresh meats and ready-to-eat products (Aggarwal *et al.*, 2000).

Michalczyk *et al.* (2012) used essential oil of coriander and hyssop directly in ground beef to inhibit growth of *Enterobacteriaceae* bacteria and undesirable sensory properties of it, especially at $6 \pm 1^\circ\text{C}$. They tested the highest sensorial acceptable concentration (0.02% v/w) of these essential oils on the microbial quality of stored ground beef. Vacuum-packed treated ground meat was stored at $0.5 \pm 0.5^\circ\text{C}$ and $6 \pm 1^\circ\text{C}$ for 15 days. Their results showed increase shelf-life up to three days by preventing undesirable sensory changes and decrease of growth of *Enterobacteriaceae* up to one to two log cycles. The reduction effect of these essential oils on total viable bacterial count and lactic acid bacteria was up to one log cycle similarly for both oils (Bakkali *et al.*, 2008).

Clove essential oil (0.1%) could significantly affect the growth of lactic acid bacteria, psychrotrophic and *Pseudomonas* spp. on raw buffalo patties at 8°C for 9 days, while could not affect growth of mesophilic bacteria (Baser & Buchbauer, 2009).

The essential oils cannot be used directly and uniformly in meat loaves. One solution for this problem is addition of essential oil in composition of food package.

Incorporation of 2, 4, 6 and 8% w/w garlic essential oil in low density polyethylene caused reduction in number of *L. monocytogenes* on ready-to-eat beef loaves during storage at 4°C , while the change in number of *E. coli* and *Brochothrix thermosphacta* were insignificant (Aguiar *et al.*, 2014).

Addition of 1, 2, 3, 4 and 5% oregano (OR) or thyme (TH) in soy protein edible films (SPEF) led to reduction in number of *E. coli*, *E. coli* O157:H7, *S. aureus*, *Pseudomonas aeruginosa* and *Lactobacillus plantarum* in ground beef stored in refrigerator. While *E. coli*, *E. coli* O157:H7 and *S. aureus* were sensitive to antimicrobial films, *L. plantarum* and *Ps. aeruginosa* showed resistance to this film. In addition, these antimicrobial films did not change total viable counts, lactic acid bacteria and *S. spp.* population significantly (Amber *et al.*, 2010).

Ravishankar *et al.* (2009) evaluated antimicrobial activity of incorporation cinnamaldehyde or carvacrol in apple-based edible against pathogenic bacteria on meat and poultry products.

Salmonella enterica or *E. coli* O157:H7 (107 CFU/g) cultures were inoculated on the surface of chicken breasts and *L. monocytogenes* was inoculated (106 CFU/g) on ham. Then inoculated products were covered with edible films incorporated with three concentrations (0.5%, 1.5%, and 3%) of cinnamaldehyde or carvacrol. The concentration of 3% of antimicrobials led to the highest reductions (4.3 to 6.8 log CFU/g) of both

S. enterica and *E. coli* O157:H7 on chicken breasts during storage at room temperature. Both of kind of essential oil and its concentration play important role in antimicrobial properties of antimicrobial film. Carvacrol showed higher activity than cinnamaldehyde in both room and refrigerator temperatures. These results were true for *L. monocytogenes* growth on ham, as well. They found no significant effect of added antimicrobials on the films physical properties (Asili *et al.*, 2009).

6.7.4 Fruits and Vegetables

Bacteria, fungi and yeasts are natural microflora of these fresh products. The dominant microflora of vegetables is soil organisms. The kind of contaminant microorganism is function of the original microflora, distribution, time, temperature, package atmosphere and other post-harvest condition (Pankaj *et al.*, 2014). On the other hand, the majority of postharvest rots of fruit happened because of fungal plant pathogens. Low pH, higher moisture content and nutrient composition of fruits make them appropriate host for growth of pathogenic fungi and produce mycotoxins (Beirão da Costa *et al.*, 2012). However, the postharvest losses of fresh product should be reduced due to limitation of resources for future generations. There have been many reports on the influences of essential oils on postharvest spoilages (Belletti *et al.*, 2007). The bioactivity of essential oils in the vapor phase makes them suitable for using as fumigants for fruits during storage.

Most of *in vitro* studies presented strong inhibitory effect of essential oils against postharvest fungi (Belletti *et al.*, 2007; Belletti *et al.*, 2008; Ben Hsouna *et al.*, 2014), but there are fewer reports on efficacy of some essential oils in improving shelf-life of fruit and vegetables.

The fungi toxic activity of mixtures of the oils may be due to synergism of two or more effective constituents in the oils that lead to decrease chance of fungi to resist against them that would be beneficial in postharvest protection (Solano & de Rojas Gante, 2012).

The antimicrobial potential of some essential oils against the native microflora of organic Swiss chard (a kind of leafy green vegetable) was studied by Ponce *et al.* (2003) using two methods:

- 1) agar diffusion technique (paper disc): a rapid semi-quantitative method to determine the microflora sensitivity to essential oils
- 2) optical method (turbidometric): a rapid and easy method to determine the MIC and MBC of different essential oils.

Results showed no significant antimicrobial effect of pinnus and *Rosa moschata*, and needs to have high concentrations of lemon, mint, rosemary, oregano and melissa oil to achieve antibacterial effects. This study presented stronger effects of eucalyptus, tea tree and clove that make them potential and useful materials as sanitisers (Pankaj *et al.*, 2014).

Botrytis cinerea Pers: Fr. (grey mold rot) can cause severe damage in many fresh plant products in pre- and post-harvest. Application of common fungicides such as benzimidazoles, dicarboximides, diethofencarb and the sterol biosynthesis inhibitors, in addition of food safety problem, lead to predominance of the pathogen resistant strains.

Several studies on the effect of essential oils against *Botrytis cinerea* have been done. Bouchra *et al.* (2003) investigated antifungal properties of the volatile compounds of seven Moroccan plants looking as new natural materials for control of plant

biodeterioration (Lopez *et al.*, 2005). They found mycelium formation was inhibited greatly with addition of *Origanum compactum* and *Thymus glandulosus*. Both of them could inhibit 100% growth of *Botrytis cinerea* at 100 ppm, when the IC₅₀s were 35.1 and 79.2 ppm, respectively because of higher amount of thymol and carvacrol as two main constituents.

Menthapulegium exhibited more poor activity at 250 ppm that could inhibit 58.5% of the mycelial growth and its IC₅₀ was 233.5 ppm (Lopez *et al.*, 2005).

Citral and citron essential oil constituents, that had showed good inhibitory effect on yeast growth in fruit beverages (Bertoli *et al.*, 2011), was used to increase the storage time of fruit-based salads. Furthermore, the organoleptic properties of fruit salads were pleasant (Ponce *et al.*, 2008).

Gutierrez, Barry-Ryan and Bourke (2009) tried to optimise the bactericidal efficacy of lemon balm, marjoram, oregano and thyme essential oils (EOs) against *Listeria* spp. and *Enterobacter* spp., *Lactobacillus* spp. and *Pseudomonas* spp. in lettuce by the agar dilution method and/or the absorbance based microplate assay. *Listeria* strains were the most sensitive bacteria, and oregano and thyme were the most effective than others. Study on different combinations of essential oils showed that oregano combined with thyme had sever effects against spoilage organisms and combination of lemon balm and thyme led to more activity against *Listeria* strains (Avila-Sosa *et al.*, 2012).

6.7.5 Others

Antifungal effect of 0, 50, 200, 350 and 500 ppm thyme, summer savory and clove essential oils were investigated in culture medium and tomato paste inoculated by *Aspergillus Xavus*. Results exhibited stronger inhibitory effect of 350 ppm thyme and 500 ppm summer savory oil on the growth of *A. Xavus*. The inhibition percent of each essential oil in tomato paste was lower than culture medium because of synergism and antagonism between components of essential oils and food constituents that can strongly affect the quantity of inhibitory activity (Başer & Demirci, 2007).

Friedman *et al.* (2004) studied antibacterial activity of 17 plant essential oils and nine effective components of essential oil on the foodborne pathogens *E. coli* O157:H7 and *Salmonella enterica* in apple juices. Oregano oil, carvacrol, eugenol, geraniol, leaf oil, cinnamon, clove bud oil, citral, cinnamon bark oil, lemongrass oil and lemon oil were most active against *E. coli*. The effective compounds against *S. enterica* were carvacrol, melissa oil, terpeineol, oregano oil, lemon oil, geraniol, lemongrass oil, citral, linalool and cinnamon leaf oil. The inhibitory effect of these materials was stronger on *S. enterica* than *E. coli* and increased with time and temperature of storage. In addition, the antibacterial effect was not changed by the acidity of the juices (Basim & Basim, 2003).

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7

Bioactivity of Essential Oils Towards Fungi and Bacteria: Mode of Action and Mathematical Tools

Antonio Bevilacqua, Barbara Speranza, Marianne Perricone, Milena Sinigaglia and Maria Rosaria Corbo

Department of the Science of Agriculture, Food and Environment (SAFE), University of Foggia, Italy

7.1 The Main Traits of Essential Oils

Although there is not a consensus towards a possible definition of essential oils (EOs), the most common one is the following: *EOs are oily aromatic and volatile liquids that can be harvested by any part of plants* (Speranza & Corbo, 2010; Burt, 2004; Böhme *et al.*, 2014). The International Organisation for Standardisation (ISO) defines an EO a *product obtained from natural raw material, either by distillation with water and steam, or from the epicarp of citrus fruits by mechanical processing, or by dry distillation* (Sadgrove & Jones, 2015).

The term 'essential oil' was introduced in sixteenth century by Paracelsus von Hohenheim, a Swiss researcher, who named *Quinta essentia* the effective component of a drug (Guenther, 1948; Speranza & Corbo, 2010). Nowadays, the two words (essential and oil) pinpoint the main attributes of EOs: they are aromatic liquid (from the Latin *essentia-ae*, *i.e.*, perfumes), soluble in oil or in lipids. Speranza and Corbo (2010) reported at least 3000 EOs, although only ca. 300 are commercially available and are currently used for different purposes (perfumes, dentistry, agriculture, food preservation, household cleaning products, natural remedies and aromatherapy).

EOs can be produced from any part of plants: leaves, flowers, peel, wood, berries, seeds, resin (Speranza & Corbo, 2010). Four protocols/modes of extraction can be used, that is, expression, fermentation, enfleurage and distillation, although the hydrodistillation is the most common approach. Flowers, leaves, wood, bark, roots, seeds are put into an alembic over a water bath (distillation apparatus); then, water is heated and the steam catches the oils. Finally, vapours flow through a cool liquid, where they are collected (Speranza & Corbo, 2010). Citrus oils are generally produced through expression, that is, citrus peel is cold-pressed due to the high amount of EOs; on other hand, if the expected amount of EO in the raw material is low, extraction with solvents or supercritical carbon dioxide should be preferred.

The composition of EOs is quite variable, depending on the raw material, mode of extraction, harvesting season of plants, weather, agronomic conditions (Burt, 2004;

Speranza & Corbo, 2010; Böhme *et al.*, 2014). Each EO generally contains 20 to 60 components, although there are major and minor components. The most important components are at 20–70% (Burt, 2004), whereas the minor compounds are in trace amounts; it is a matter of debate if the biological activity of EOs relies on the major components or if minor compounds also play a role (Burt, 2004). Speranza and Corbo (2010) reported some examples of major components of EOs, that is, carvacrol (30%) and thymol (27%) from *Origanum compactum*; linalol (68%) from *Coriandrum sativum*; menthol (59%) and menthone (19%) from *Menthapiperita*. Nowadays, the term EOs is used to refer to the whole mixture, as well as to the major components of the mixture.

The components of EOs can be either terpenes/terpenoids or terpenes-derived molecules and the molecules from phenylpropane. Isoprene (C5) is the basic constituent of terpenes (hemiterpenes-C5; monoterpenes-C10; sesquiterpenes-C15; diterpenes-C20; triterpenes-C30; tetraterpenes-C40). A terpene-containing oxygen is called terpenoid. Monoterpenes represent up 90% of EOs and comprise carbures (myrcene, ocimene, p-cimene, phellandrenes, camphene, sabinene, *etc.*), alcohols (geraniol, linalol, citronellol, lavandulol, nerol, menthol, fenchol, *etc.*), aldehydes (geranial, neral, citronellal, *etc.*), ketones (menthones, carvone, camphor, pino-carvone, *etc.*), esters and ethers, peroxides and phenols (thymol, carvacrol, *etc.*) (Speranza & Corbo, 2010).

Terpenoid EOs are produced via mevalonate pathway, involving the derivatisation and polymerisation of five-membered isoprene alkenes from IPP (isoprenyldiphosphate) and DMAPP (dimethylallyldiphosphate) (Sadgrove & Jones, 2015).

Molecules from phenylpropane are less frequent; they are made by aldehydes (cinnamaldehyde), alcohols (cinnamic alcohol), phenols (chavicol, eugenol), methoxy derivatives and methylene dioxy compounds. Phenylpropanoids are synthesised from the shikimic acid, via phosphoenol pyruvate and erythrose-4 phosphate (Sadgrove & Jones, 2015).

Another possible classification of the major components of EOs was reported by Sadgrove and Jones (2015), based on the aromatherapy classification of the functional groups (Schnaubelt, 1995); thus, EOs can be:

- alcohols and phenols (*e.g.*, α -terpineol);
- phenols (thymol);
- ketones (eucarvone);
- acids (cinnamic acid);
- coumarins (umbelliferone);
- esters (exyl propionate);
- phthalides (3-butylphthalide);
- methyl ethers (methyl eugenol);
- cyclic ethers (1,8-cineol);
- furans (methofuran);
- lactones (jasmolactone);
- aldehydes (cinnamaldehyde).

Table 7.1 offers a brief overview of the possible use of EOs; the focus of this chapter is quite different, thus in the following sections there is a focus on the mode of action of EOs towards bacteria and fungi and on the mathematical tools to determine this effect.

Table 7.1 Possible uses of EOs.

When/where	How
Food preservation (Speranza & Corbo, 2010)	Antibacterial and antifungal activity in a wide variety of foods (juices, dairy products, fish, meat, salads); the effect could be enhanced by the hurdle approach
Phytotherapy (Harris, 2016)	Acaricidal activity, anticarcinogenic, treatment of propionibacteria and methicillin-resistant <i>Staphylococcus aureus</i> , anticandida properties, antiviral activity, controlling microflora of oral cavity and in atopic dermatitis, treatment of functional dyspepsia, possible effects in the irritable bowel disease, pain relief (headache, dysmenorrhea, infant colic, pruritis), pediculicidal activity, pains of the respiratory tracts, allergic rhinitis.
Aromatherapy (Lis-Balchin, 2016)	Massage, diffusion of EOs, internal usage. There are some challenges related to possible false claims or to a placebo effects. Possible toxicity.
Aroma-vital cuisine (Kettenring & Vucemilovic-Geeganage, 2016)	Aroma-vital cuisine is based upon the addition of some basic oils or mixtures of oils to salads, main dishes and desserts: <i>Euro Asia</i> (lime, coriander seed, ginger, lemongrass, green pepper); <i>O Sole Mio</i> (thyme, rosemary, clementine); <i>Capri</i> (orange, lemon); <i>Bergamot-Grand Manier</i> (grapefruit, orange, lemon, bergamot); <i>Magic Orange</i> (orange, vanilla extract, cacao extract, rose); <i>Clary Sage and Bergamot</i> (clary sage, bergamot); <i>Peppermint</i> (peppermint); <i>Lavender</i> (lavender). Fragrances are a kind of soul food, as the information scent can be perceived in every section of our self, physical, energetic as well as intellectual, from a holistic point of view. Adding spices with essential oils according to Aroma-vital cuisine combines sensually with sanative potential.
Veterinary medicine (Can Başer & Franz, 2016)	Oils attracting or oils repelling animals, insecticidal, pest and antiparasitic oils, animal feed, treating animal diseases (expectorant, teeth hygiene, intestinal worms).
Psychopharmacology (Nunes <i>et al.</i> , 2016)	Improvement of visual attention, controversial effect on memory, possible effect of citrus on lexical decision task, achievement of higher monetary goals and making more concessions.

7.2 Antibacterial Activity of EOs

The antimicrobial activity of EOs is probably linked to their hydrophobicity, responsible of the increase of cell permeability and loss of cellular components. The final results could be the lysis, and the death. The outer membrane of gram-negative represent an effective hurdle, thus gram-positive bacteria are more sensitive to EOs (Böhme *et al.*, 2014). Speranza and Corbo (2010), based on many literature reports, suggested a possible hit of susceptibility to EOs as follows: fungi > gram-positive bacteria > gram-negative bacteria. Some EOs can also affect gram-negative microorganisms, but there two species (*Klebsiellapneumoniae* and *Pseudomonas aeruginosa*) showing a high resistance (Böhme *et al.*, 2014). *K. pneumonia* could be inhibited only by *Rosmarinus officinalis*, *Nepetacataria*, *Menthalongifolia*, *Menthaviridis* and *Monticaliaandicola*.

Speranza and Corbo (2010) suggested some key elements for the antimicrobial activity of EOs, that is:

- 1) different EOs possess a different bioactivity;
- 2) the chemical composition, and secondary groups;
- 3) the mode of action of some EOs is still unclear;
- 4) the susceptibility of microorganisms to EOs bioactivity is quite different;
- 5) high amounts of EOS should be used in foods (1–3%);
- 6) fats, carbohydrates, proteins, water, salt, antioxidants, preservatives, other additives and pH can increase or decrease the effect of EOs in foods;
- 7) fats and/or proteins protect bacteria;
- 8) salt can enhance the action of EOs;
- 9) the physical structure of a food might limit the antibacterial activity of EOs.

An extensive review of the effect of some food components on the bioactivity of EOs can be found in Speranza and Corbo (2010), Böhme *et al.* (2014) and Perricone *et al.* (2015). This section offers an overview on the antibacterial activity of EOs with a focus on the most important effects at cell level. A brief synopsis of the section can be found in Figure 7.1.

7.2.1 Effect on Cell Morphology

First macroscopic effect of the bioactivity of EOs towards bacteria is the change of cell shape at microscope, as well as the coagulation of cytoplasm, as revealed by SEM (scanning electron microscopy), TEM (transmission electron microscopy) and ATM (atomic force microscopy) (Hyldgaard *et al.*, 2012). These effects (described as ‘crumpled or collapsed cells’) are the signs of the disruption of the outer or inner membrane, as well as of the coagulation of inner content.

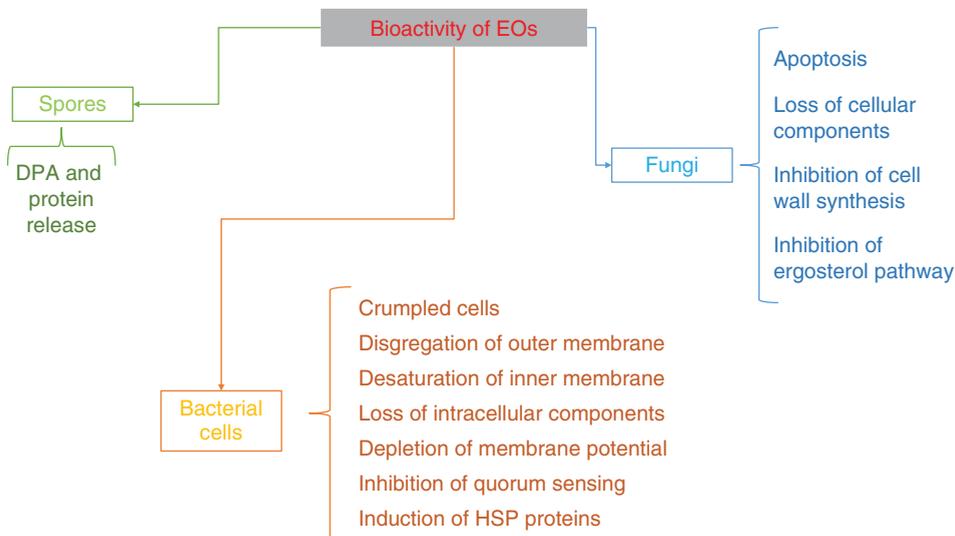


Figure 7.1 Mode of action of EOs against bacteria and fungi.

7.2.2 Disruption of the Outer Membrane of Gram-Negative Bacteria

The outer membrane is an efficient permeation control (Benson, 1998; Nikaido 2003; Fadli *et al.*, 2012); it regulates and protects the inner membrane from via the proteinaceous channels (Weiss *et al.*, 1984; Vaara, 1992; Pagès *et al.*, 2008; Fadli *et al.*, 2012).

It limits the bioactivity of EOs towards gram-negative bacteria; however, some EOs can act on it and cause its disruption. The first sign of the damage of the outer membrane is the release of LPS (Helander *et al.*, 1998); however, Fadli *et al.* (2012) found a release of β -lactamase or β -galactosidase, due to an increased permeabilisation of both outer and inner membranes without a detectable degradation suggested that some EOs (e.g., *Thymus maroccanus* EO) could permeabilise both OM and IM but without causing detectable degradation of cellular constituents. It seems that they create discontinuities through the membrane allowing the release of proteins such as.

7.2.3 Effect on the Cytoplasmatic Membrane

Antimicrobial compounds that act on the membrane can cause depolarisation or increased permeability through various mechanisms. For example, some peptides form pores (Cotter *et al.*, 2005; Fantner *et al.*, 2010) while EOs have a fluidifying effect on the membrane (Trombetta *et al.*, 2005; Cristani *et al.*, 2007). Many authors reported the ability of EOs to impair the membrane, as suggested by the efflux of K^+ , Ca^{2+} , nucleic acids and proteins (Carson *et al.*, 2002; De Souza *et al.*, 2010; Rao *et al.*, 2010; Somolinos *et al.*, 2010).

EOs also induced a significant effect on the qualitative composition of fatty acids. Siroli *et al.* (2015) evaluated the effect of thyme and oregano essential oils, carvacrol, thymol, citral and trans-2-hexenal on membrane fatty acid composition of *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enteritidis* and found that EOs caused an increase of the amounts of unsaturated fatty acids, and *trans*-isomers. Previously, they had found a similar effect after the exposition to sub-lethal concentrations of hexanal and 2-(E)-hexenal (Patrignani *et al.*, 2008).

7.2.4 Homeostasis, Enzymes and Other Activities

EOs act at different levels on cells; generally, the disruption of the membrane causes the uncontrolled leakage of some cellular constituents. Turgis *et al.* (2009) tested the EO from mustard on *E. coli* O157:H7 and *Salmonella typhi* and found a release of ATP from cells, as well as a reduction of intracellular pH. This effect was also found in *Vibriocholearae* (Sanchez *et al.*, 2010), *L.monocytogenes* (Oussalah *et al.*, 2006), *Bacillus cereus* and *Enterococcus faecalis* (Silva *et al.*, 2011).

EOs can also impair the respiratory efflux, and/or cause the depletion of membrane potential (Cox *et al.*, 2000; Ultee *et al.*, 2002; Fitzgerald *et al.*, 2004; Xu *et al.*, 2008; Fisher & Philips, 2009; Bouhdid *et al.*, 2009, 2010; Silva *et al.*, 2011).

In addition, there are some evidences on a possible effect of EOs on some specific enzymes, like urease, proteases, alcohol dehydrogenase, thioredoxinreductase, acetate kinase, carboxylases, α -amylases, subtilisin, acetyl-CoA-enzyme forming systems, ATPase (Thoroski, 1989; Focke *et al.*, 1990; Wendakoon & Morihiko, 1995; Rabinkov *et al.*, 1998; Gill & Holley, 2006; Derakhshan *et al.*, 2008; Luciano & Holley, 2009).

Recently, some authors suggested that EOs could play a significant role in biofilm formation, in quorum-sensing (QS) and virulence attributes. QS is a key factor for pathogenicity and antibiotic resistance, as it affects the production of acylated homoserine lactones (AHLs) and consequently motility, swarming, biofilm production, and antibiotic resistance (Szabò *et al.*, 2010).

Sepahi *et al.* (2015) studied the effect of two EOs from *Apiaceae* family towards *Ps. aeruginosa* and found that in some cases biofilm production was significantly reduced; moreover, the expression analysis of QS and virulence genes showed that they were down-regulated. The anti-QS activity of some EOs was also suggested by Khan *et al.* (2009) for clove oil against *Ps. aeruginosa* and *Chromobacterium violaceum*, and for rose, geranium, lavender and rosemary against *E. coli* and *Chr. violaceum* (Szabò *et al.*, 2010).

7.2.5 Changes in Proteome and Transcriptome

Some authors reported many evidences on a possible activity of EOs on the whole proteome and transcriptome. Di Pasqua *et al.* (2010) studied the effect of sublethal concentrations of thymol on — a strain of *Salmonella enterica* serovar Thompson; through protein extraction and analysis by SDS-PAGE and MALDI-TOF, they found that treated cells did not express thioredoxin-1, whereas different chaperons or outer membrane proteins were up-regulated. In addition, some other proteins (*e.g.*, GroEL and DnaK, key proteins in the protection mechanism towards thermal stress) were synthesised by treated cells but not by the control ones. Finally, thymol impaired citrate pathway, as well as some other enzymes involved in the synthesis of ATP. The over-expression of GroEL was also reported by carvacrol in *E. coli* O157:H7; on the other hand, treated cells did not produce flagellin (Burt *et al.*, 2007).

Gomes Neto *et al.* (2015) suggested that these changes could be mediated by sigma factors $\sigma(S)$ (RpoS) and $\sigma(B)$ (SigB), involved in the general stress response; mutant strains not possessing these factors, in fact, were more sensitive to EOs than the wild isolates. EOs probably induced an up-regulation of HSP60 and HSP70 proteins and caused a shift of the proteome towards an increased general stress response (Burt *et al.*, 2007; Di Pasqua *et al.*, 2010). This idea could be confirmed by the evidence that *Thymus maroccanus* essential oil induced significant modification in porins (influx) AcrAB family (efflux of various drugs). These medications induced an increased resistance to several antibiotic families (Fadli *et al.*, 2014). These effects could be a threat for the use of EOs in food preservation as well as in phytotherapy, as they could induce the emergence of resistant strains; however, this drawback was found only at sub-lethal concentrations but not at MICs (minimal inhibitory concentration).

7.2.6 EOs and Bacterial Spores

A wide variety of EOs was tested towards spore-forming bacteria (*Alicyclobacillus* spp., *Bacillus* spp., *Clostridium* spp.) and for many EOs it is possible to find MIC; however, it is not clear how EOs act on spores. Lawrence and Palombo (2009) examined the spores of *Bacillus* after the exposure to several EOs and suggested that leakage of spore contents was responsible of the sporicidal action. Bevilacqua and colleagues (unpublished results) tested the effect of a commercial lemon extract on the spores of *Alicyclobacillus acidoterrestris* and found that treated spores released DPA and proteins.

7.3 Antifungal Activity of EOs

EOs were successfully used to control and/or inhibit fungi, with a wide variety of effect, like the prolongation of the shelf-life of fruit and vegetables, the inhibition of candidiasis and the reduction of toxin production (Böhme *et al.*, 2014). However, few authors addressed how EOs act on fungi. Some (and few) key points are the following ones; probably each fungus can be inactivated by some or by all the reported mechanisms.

- Apoptosis: Ferreira *et al.* (2014) studied the effect of the EO of *Menthapiperita* against some strains of *Saccharomyces cerevisiae* and found that the cytotoxicity of the oil was associated with the increase of ROS amounts (reactive oxygen species), mitochondrial fragmentation and chromatin condensation.
- Hydrogen bond formation: Daferera *et al.* (2000) found that the antifungal effect of EOs was the result of hydrogen bonds between the hydroxyl groups of phenolic compounds and the active sites of cellular enzymes.
- Loss of cellular integrity: EOs could cause an injury on the cell wall, with a loss of cytoplasmic constituents (Sharma & Tripathi, 2006).
- Other effects: Lucini *et al.* (2006) suggested that fungi are inhibited by monoterpenes, which could lead to the production of lipid peroxides, such as hydroxyl, alkoxyl and alkoperoxy radicals. Allicin (isolated from garlic) could inhibit the enzymes with -SH groups, and affect the synthesis of fatty acids, lipids, DNA or RNA (Beuchat & Golden, 1989). EOs could also inhibit the synthesis of cell wall via the inhibition of β -(1–3)-glucan and chitin synthase (Bang *et al.*, 2000; Hyldgaard *et al.*, 2012) and the disruption of the biosynthesis of ergosterol (Ahmad *et al.*, 2011).

7.4 Mathematical Tools

The antimicrobial effect of EOs, as well as of other chemicals or by physical approaches, can be evaluated by using a modified micro-dilution or by evaluating the growth/inactivation through plating. The approaches can be different for bacteria/yeasts and filamentous fungi (moulds); the following sections focus on the most important tools, whereas the last paragraph deals with the evaluation of the combined use of two EOs/compounds (eqn. 7.1).

7.4.1 Bacteria and Yeasts

The bioactivity of EOs towards bacteria and yeasts can be evaluated in the same way. A widely used approach *in vitro* is absorbance reading of lab media containing different amounts of the active compounds; this approach can be considered a modified micro-dilution. Blaszyk and Holley (1998) first proposed it and suggested the calculation of a growth index (GI), as follows:

$$GI = \frac{(A - NC)}{PC} * 100 \quad (\text{eqn. 7.1})$$

where A is the absorbance of the sample with the active compound, PC the positive control (sample without EOs) and NC the negative control (un-inoculated sample with EOs).

This is a time-dependent index and can be used to evaluate the bioactivity of the active compound for each time (see eqns. 7.2 and 7.3). Falcone *et al.* (2005) slightly modified this approach and used the growth index to evaluate the minimal inhibitory concentration (MIC) or the not-inhibitory concentration (NIC) of thymol towards a wide variety of bacteria and yeasts (see Figure 7.2 for a definition of MIC and NIC).

They used GI as dependent variable whereas thymol concentration (T) was the independent factor in the equation of Lambert and Pearson (2000):

$$GI = \exp \left\{ \frac{\left[\frac{T}{\ln MIC} \right]^{-\left\{ \frac{\ln \ln NIC}{\ln MIC} \right\}}}{\exp \left(-\frac{\ln \ln NIC}{e} \right)} \right\} \quad (\text{eqn. 7.2})$$

The growth index was slightly modified by Bevilacqua *et al.* (2009) as follows:

$$GI = \frac{A}{PC} * 100 \quad (\text{eqn. 7.3})$$

This modified index was first used to model the effect of salt on yeasts and bacteria from olives and then proposed to assess the antibacterial effect of some natural compounds.

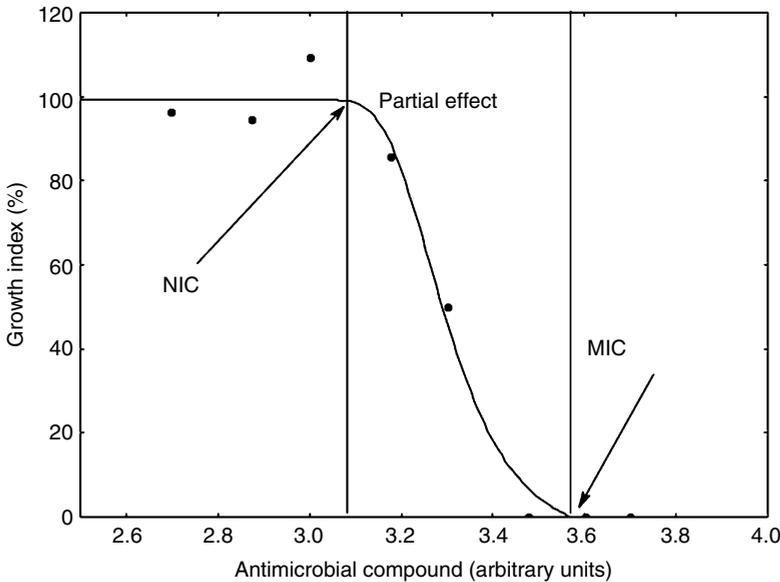


Figure 7.2 Bioactivity of an extract. Definition of MIC and NIC.

The index should be read as follows:

GI < 25%: complete inhibition of the test microorganism.

25% < GI < 75%: partial inhibition

GI > 75%: growth similar to the positive control.

As reported for the GI from Blaszyk and Holley (1998), GI is time-dependent and offers a static approach, that is, a picture of the microbial population for a particular time (see eqn. 7.4). A modified index from the GI is the inhibition index (II), which offers the same information of GI but in a negative perspective (Bevilacqua *et al.*, 2014):

$$II = \frac{(PC - A)}{PC} * 100 \quad (\text{eqn. 7.4})$$

An alternative approach relies upon the use of the primary fitting curve (growth — Gompertz, Baranyi, logistic, or inactivation — Weibull, shoulder-tail, *etc.*) to model the whole trend of the population over time and use a fitting parameter (for example the lag time or the growth rate for the growth models, or d-value, death rate for inactivation functions) to pinpoint the bioactivity of the extract.

Both inactivation and growth models can be used for the data from plating, whereas the data from spectrophotometry can be fitted only by growth function (see eqn. 7.5).

For this purpose, Bevilacqua *et al.* (2010) modified the Gompertz function as follows:

$$y = k + A * \exp \left\{ -\exp \left[\left(\mu_{\max} * 2.7182 \right) * \frac{(NST - t)}{A} + 1 \right] \right\} \quad (\text{eqn. 7.5})$$

In this equation the classical parameter λ (lag phase) was named no spoiling time (NST), that is, the time before population attained a critical level of 6 log cfu/ml and caused a significant change in the absorbance of the medium.

7.4.2 Filamentous Fungi

The bioactivity of many chemicals *in vitro* is evaluated through a modified plate assay and the diameter of the fungal colony was used as an index of the bioactivity of the antimicrobial compound, compared to the positive control.

A possible approach is the use of a Gompertz or a logistic-like function (the model of Dantigny) and evaluate a critical time to attain a break-point in the fungal diameter (eqn. 7.6).

Altieri *et al.* (2007) proposed the minimum detection time, that is, the time to attain a fungal colony of 1 cm.

$$y = 1 - A \cdot \exp \left\{ -\exp \left\{ \left[\left(\mu_{\max} \cdot 2.71 \right) \cdot \frac{\lambda - MDT}{A} \right] + 1 \right\} \right\} + \\ + A \cdot \exp \left\{ -\exp \left\{ \left[\left(\mu_{\max} \cdot 2.71 \right) \cdot \frac{\lambda - t}{A} \right] + 1 \right\} \right\} \quad (\text{eqn. 7.6})$$

In the above-mentioned equation y is the colony diameter (cm); A the maximum colony diameter attained within the experimental time (cm); μ_{max} the maximal radial growth rate (cm/time units); λ the lag value (time units), t is the time and MDT, the *minimum detection time*.

The parameter λ could be assumed as the time after which the radial growth of the fungal colony started (eqn. 7.7).

Bevilacqua *et al.* (2017) slightly modified the equation of Dantigny *et al.* (2011), cast in the following form:

$$D = \frac{D_{max}}{1 + \exp[k(\tau - t)]} \quad (\text{eqn. 7.7})$$

Where D is the diameter of the fungal colony over time; D_{max} , the maximum diameter of fungal colony; τ , the time to attain a $\frac{1}{2}$ of D_{max} and t the time (see eqn. 7.8).

In addition, a modified GI approach was proposed by Bevilacqua *et al.* (2012) to model the effect of citrus extract on *Fusarium oxysporum* inoculated in pineapple juice.

For each time of storage data were modelled as growth index, as follows:

$$GI = \frac{[C]_{ant}}{[C]_{ctr}} \cdot 100 \quad (\text{eqn. 7.8})$$

where $[C]_{ant}$ is *F. oxysporum* in the sample containing the antimicrobial and $[C]_{ctr}$ is the population level in the control (see eqn. 7.9).

For each time of analysis GI values were modelled as a function of the concentration of antimicrobial through a negative Gompertz equation, modified by Zwietering *et al.* (1990) and cast as follows:

$$GI_{C^*} = GI_i - \Delta GI_{max} \exp \left\{ -\exp \left[\frac{d_{GI} e}{\Delta GI_{max}} (NIC - C) + 1 \right] \right\} \quad (\text{eqn. 7.9})$$

where GI_{C^*} is the dependent variable; GI_i the growth index in the control (ca. 100%); ΔGI_{max} the maximum decrease of GI attained at the MIC (minimum inhibitory concentration); d_{GI} , the decrease of GI for each unit of concentration of the antimicrobial compound; NIC , correspondent to the shoulder phase of the curve and assumed as the not-inhibitory concentration of the compound; C , the concentration of the antimicrobial (see eqn. 7.10).

Through the fitting parameters of Gompertz equation, MIC was evaluated as follows:

$$MIC = \frac{\Delta GI_{max}}{e * d_{GI}} + NIC - \frac{\Delta GI_{max} * \ln \left[-\ln \left(\frac{GI_0 - 0.2}{\Delta GI_{max}} \right) \right]}{e * \mu_{max}} \quad (\text{eqn. 7.10})$$

Where 0.2 is the detection limit of *F. oxysporum* through the spread plate method (1 log cfu/ml).

7.4.3 Fractional Inhibitory Index

Many times EOs are used as mixtures; thus, Gutierrez *et al.* (2009) proposed a combined index and described two fungal parameters: MIC (minimal inhibitory concentration with a fungistatic effect) and MFC (minimum fungicidal concentration with a killing effect; see eqn. 7.11). When testing the antifungal activity of a blend/mixture of EOs, the effect is expressed over a fractional inhibitory concentration index (FIC_{index}):

$$FIC_{index} = \frac{MFC_{Amix}}{MFC_A} + \frac{MFC_{Bmix}}{MFC_B} \quad (\text{eqn. 7.11})$$

Where MFC is the minimal fungicidal concentration (individual concentration or concentration of mixtures A and B).

The index can be used as follows:

$FIC < 0.5$: synergistic effect

$0.5 < FIC < 1$: additive effect

$1 < FIC < 4$: indifference

$FIC > 4$: antagonism

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8

Antioxidant Activity of Essential Oils in Foods

Seyed Mohammad Bagher Hashemi, Shima Bazgir Khorram and Maryam Sohrabi

Department of Food Science and Technology, College of Agriculture, Fasa University, Fasa, Iran

8.1 Introduction

Most of the fats found in food are in the form of triglyceride that is a proper source for oxidation and unfavourable taste. Spontaneous reactions of atmospheric oxygen with lipids are known as autoxidation that is an important and common process for oxidation deterioration. Unsaturated fatty acids have the potential to be destructed by this process by free fatty acids, triacylglycerol or phospholipids. When light and sensitizer like chlorophyll exist, transforms atmospheric oxygen to singlet oxygen, which plays an important role in oxidation deterioration. Metals like iron, copper and enzymes like lipoxygenase play important roles in initiation and speed up of this process (Schaich, 2005).

Antioxidants are compounds that protect fats and other alimentary components like proteins from oxidation. Although using antioxidants in alimentary components dates back to ancient days, in which plants and spices were used in foods, using synthetic antioxidants dates back 60 years. Many synthetic antioxidants used in foods are investigated, amongst which, TBHQ, BHT, BHA and PG have wider usage in alimentary material. Researchers are trying to identify better and more suitable antioxidants to be used in foods (Yanishlieva & Maslarova, 2001). Synthetic antioxidants are added to foods directly and indirectly, for example to the food package. All antioxidants have positive and negative points, so special antioxidants must be found considering these points, that is, thermal stability, effective concentration and synergistic effect. Of course, monitoring rules must not be overlooked, especially for those antioxidants, which have unfavourable effects on health. Synthetic antioxidants go under safety and toxicology experiments in the least concentration, while the permission to use them differs from one country to another and it usually depends on alimentary material and other considerable conditions. Phenolic and menophenolic compounds have a wide variety of usages in foods. Different structures of these compounds result in different physical attributes and antioxidant activity. BHA and BHT are amongst these compounds, which are used in foods. Researches have shown that these two compounds contain toxic attributes and can bring about unfavourable side effects for consumers, like renal and pulmonary complications. Indeed,

these cases are denied in some other researches and anti-tumour traits have been given to them. In addition, some researchers have other opinions and believe that these experiments are done on rat and cannot be generalised to men. For instance, the toxic concentration for human is 1500 times more than toxic concentration for rat. Despite these positive and negative points, these two compounds are allowed to be used in many countries. The highest concentration for them is 0.02% that is determined based on the fat concentration in that alimentary material. However, these two antioxidants have antioxidant activity in low concentrations and can play peroxidation role in high concentrations. Their consumption rule differs in each food, for example, they can't be used in sea foods (Wanasundaea & Shahidi, 2001).

Being concerned about unfavourable effects of using synthetic antioxidants, have increased consumers' tendency to use natural antioxidants, because these compounds are not only safe but also can have effective nutritive effects. Compounds made of plants have high potential for these purposes, amongst which we can mention plant extracts and their extracted essential oils. Essential oils are natural, complicated compounds with heavy odour. They are liquid, volatile, rarely chromatic, and soluble in fat and organic solvents, and their density is generally lower than water. Essential oils are made from plants, which usually live in hot areas like Mediterranean and Equator areas (Bakkali *et al.*, 2008). They are synthesised by different parts of plants and aerial organs, seed, flower and fruit are amongst them. Essential oils can be used as useful antioxidants in foods; however, using these natural compounds can cause some problems like less antioxidant effect comparing to other synthetic compounds and unfavourable taste and odour, in some cases (Yanishlieva & Maslarova, 2001).

8.2 *In Vitro* Antioxidant Activity of Essential Oils

Experiments used for *in vitro* investigation of antioxidant activity of essential oils usually include 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH), ferric reducing-antioxidant power (FRAP), β -carotene bleaching assay and ABTS radical scavenging activity. In a study, the effect of three factors, that is, pressure, temperature and time on super critical extraction of CO₂, performance, chemical compound and antioxidant effect of lavender essential oil was studied. Time and pressure had a significant effect on antioxidant activity of essential oil, while temperature had less significant effect (Shukla *et al.*, 2012). Essential oils acquired from aerial organs of annual plants were analysed using GC-MS. More than 100 compounds are found, which indicate 95.5% of all essential oils' compounds. The major components of essential oils include oxygenated monoterpene, artemisia ketone (30.7%) and camphor (15.8%). Segregated essential oils are tested for radical scavenging activity using DPPH, ABTS, ORAC and ferrozine tests. Essential oil didn't show significant antioxidant activity in any test; however, it was comparable to thymol, a well-known antioxidant (Cavar *et al.*, 2012). The film made of fish-scale gelatin combined with lemon essential oil showed high antioxidant activity in ABTS and FRAP tests, and this antioxidant activity was higher comparing to bergamot (Tongnuanchan *et al.*, 2012). Essential oils extracted from fruits (*Foeniculum vulgare* var. *dulce*, *Foeniculum vulgare* var. *azoricum* and *Foeniculum vulgare* var. *vulgare*) were studied for chemical compound and antioxidant activity. GC-MS showed 18 monoterpene in each essential oil of all three fruits, but the percentages in these three types are very

different. Estragole, trans-anethole, limonene and fenchone were abundant in all investigated essential oil. The result of antioxidant tests indicated high antioxidant activity of fruits (Shahat *et al.*, 2011). Antioxidant activity of essential oils of Amomumtsao-KO indicated that these essential oils have weak antioxidant activity (Yang *et al.*, 2010a). *O. glandulosum* was collected from three locations in Tunisia and their essential oils' chemical composition and antioxidant activity was evaluated. Main components of essential oils are thymol, *p*-cymene, carvacrol and γ -terpinene. Radical scavenging ability (DPPH) in IC₅₀ range was 59–80 mg/L. The correlation between total phenol content of essential oils and DPPH radical absorbent capacity was detected (Mechergui *et al.*, 2010). Antioxidant activity of essential oils in plants and fruits including lavender, mint, *Rosmarinus officinalis*, lemon, grape fruit and frankincense was compared. The results indicated that antioxidant activity of lavender essential oils was more than other plants (Yang *et al.*, 2010b). Essential oils of Coriander and *Carum carvi* L. fruit showed radical scavenging ability against DPPH radicals (Samojlik *et al.*, 2010). Essential oil of anise, cardamom, cinnamon, zedoary, pikhom and ginger indicated significant antioxidant activity in DPPH and FRAP tests. These essential oils also showed high total phenolics (Nanasombat & Wimuttigosol, 2011). Comparing the antioxidant activity of essential oils and *Rhizoma homalomenae* extract indicated that the extract of this plant has higher antioxidant activity comparing to its essential oil (Zeng *et al.*, 2011). Using DPPH test showed that *Semenovia tragioides* essential oils had weaker antioxidant activity. The major component of essential oils was lavenderyl acetate (Bamoniri *et al.*, 2010). Essential oils extracted from plant corm showed significant antioxidant activity that was more than ascorbic acid (Gourine *et al.*, 2010). *Bunium persicum* Boiss is economically a medicinal and important plant, which lives as a wild plant in arid parts of Iran. The main components of this plant's essential oil include caryophyllene (27.81%), γ -terpinene (15.19%) and cumyl stearate (14.67%). Investigating the antioxidant activity of this plant was done using DPPH and β -carotene bleaching tests. In DPPH system, the amount of IC₅₀ essential oils was calculated 0.88 mg/ml. Antioxidant activity of essential oil (0.45%) in β -carotene is almost equal to BHT 0.01% (Shahsavari *et al.*, 2008). Chemical composition of essential oil extracted from *Teucrium bisabolone* included isocaryophyllene (20.24%), santalene (11.27%), sesquiphellandrene (14.73%), caryophyllene (7.18%) and dolichodial (9.38%) that showed significant antioxidant activity in DPPH test (Ricci *et al.*, 2005). Antioxidant activity of essential oil from two species *Achillea millefolium* showed significant antioxidant activity in DPPH test (Bozin *et al.*, 2007). *Teucrium sauvagei* essential oil showed proper antioxidant activity in DPPH and ABTS tests (BelHadj Salah *et al.*, 2007). Essential oil of leaf and flower of *Bidens pilosa* showed proper antioxidant activity in DPPH test (Deba *et al.*, 2008). *Thymus* and *Origanum* essential oil showed antioxidant activity that depends on chemical composition of essential oils and selecting method for such an activity (Hazzit *et al.*, 2008). Essential oil of *Thymus caespititius*, *T. capitellatus*, *T. camphoratus*, *T. carnosus*, *T. sylvestris*, *T. pulegioides* and *T. zygis* collected from different areas of Portugal, were investigated. Results indicated their proper antioxidant activity (Dandlen *et al.*, 2010). Antioxidant activity of essential oils, oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*), sage (*Salvia officinalis*) and clove (*Salvia Syzygiumaromaticum*) and their total phenol composition was measured. Clove essential oils had the highest total phenol (898.89 mL/L GAE) and the highest DPPH radical inhibition percent (98.74%) and the highest amount of FRAP (1.47 TEAC).

Thyme essential oils showed the highest TBARS inhibition percent (89.84%). All the essential oils under investigation were able to chelate the iron (II) and rosemary essential oils had the highest effect (76.06%) in this regard. Oregano essential oils showed the highest antioxidant activity in Rancimat (Viuda-Martos *et al.*, 2009). *Foeniculum vulgare* Mill essential oils had the radical scavenging ability up to IC_{50} : 32.32 (mg/mL) (Anwar *et al.*, 2009). Antioxidant activity of essential oils separated from aerial organs of Tunisian *Thymus capitatus* was investigated by FRAP, DPPH and TBARS methods and was compared to BHA and BHT. In FRAP method, essential oils and synthetic antioxidants didn't show antioxidant activity; however, in other methods, essential oils showed better antioxidant activity comparing to synthetic antioxidants. The major components of essential oils were carvacrol (62%–83%), *p*-cymene (5%–17%), γ -terpinene (14%–2%) and β -caryophyllene (4%–1%) (Bounatirou *et al.*, 2007). *C. libanoticum* essential oils showed radical scavenging ability ($IC_{50} > 30$) (Demirci *et al.*, 2007). Antioxidant activity of *Rosmarinus officinalis* essential oils is performed using DPPH and β -carotene tests, comparing to three compositions: β -pinene, α -pinene and 1,8-cineole. Results indicated that essential oils of this plant have more antioxidant activity comparing to these compositions (Wang *et al.*, 2008). *Ocimum basilicum* L. essential oils showed proper antioxidant activity, which in part relates to harvest season of this plant (Ijaz Hussain *et al.*, 2008). Antioxidant activity of blackseed essential oil was appropriate that was less than that of rosemary extract. It is reported that this higher antioxidant activity was due to higher amounts of phenol in rosemary extract comparing to blackseed essential oils (Erkan *et al.*, 2008). *Thymus caramanicus* essential oils showed proper antioxidant activity that was less than this plant's extract. Main components of essential oils included: thymol (3.3%), carvacrol (85.9%), *p*-cymene (3.2%), γ -terpinene (1.8%) and borneol (1.3%) (Safaei-Ghomi *et al.*, 2009). *Origanum syriacum* and *O. ehrenbergii* essential oil collected in Lebanon showed significant power in scavenging DPPH radicals (Loizzo *et al.*, 2009). Antioxidant activity of essential oils extracted from parsley (*Crispum petroselinum*) was examined by three different methods: DPPH, β -carotene and chelating Fe^{2+} . Results indicated that parsley essential oils have especial degree of antioxidant activities regarding β -carotene and DPPH, but it showed low chelating capacity (Zhang *et al.*, 2006). Antioxidant activity of pink grapefruit (*Citrus paradise* L.), lemon (*Citrus limon* L.), clove buds (*Caryophyllus aromaticus* L.) and coriander (*Coriandrum sativum* L.) essential oil was studied. The highest and lowest antioxidant activity relates to grapefruit and clove bud, respectively (Misharina & Samusenko, 2008). Essential oil extracted from *Origanum glandulosum* Desf was investigated for antioxidant activity. Essential oils collected from different areas showed high degree of antioxidant activity (Ruberto *et al.*, 2001). Generally, *in vitro* investigations of antioxidant activity have some limitations including not considering factors like physical situation of antioxidant and environmental conditions. Therefore, to attain reliable results it is better that antioxidant activity of essential oils is also investigated in desired food.

8.3 Edible Oils and Fats

Edible oils are sensitive to the process of oxidation. Oxidative reactions cause unpleasant flavour and taste and produce toxic compounds in these products. Antioxidants were the compounds that could prevent oxidative reactions and increase shelf-life of the oils.

In recent years, adding essential oil to edible oils was increased due to having proper antioxidant activity and being natural. In this part, we investigate the previous researches conducted about using essential oils as antioxidants in edible oils.

Preventing the oxidation of methyl esters of fatty acids by oregano essential oil was studied using capillary gas chromatography. A combination of fatty acids containing saturated and unsaturated, mono, di and poly (unsaturated fatty acid with one, two or many dual bonds) with 16 to 24 carbon atoms was used. Alteration in the compound of esters, in both of the samples made by oregano essential oil and without it, was tested for one year during autoxidation and in presence of light. It was revealed that the oxidation rate of unsaturated fatty acids was enhanced because of increase in their unsaturation degree. Oregano essential oil inhibited the oxidation process and antioxidant activity of essential oil increased by increase in concentration. The results also indicated that thymol and carvacrol were the major antioxidant compounds of oregano oil, and the antioxidant activity could be referred to these compounds (Terenina *et al.*, 2011). In another study, the possible effect of blackseed (*Nigella sativa* L.) essential oil on stability of sunflower oil was investigated under accelerated storage. The results showed that essential oil could stabilise the sunflower oil to a certain extent. The analysis of evaluating the amount of peroxide, *p*-anisidine, non-oxidised linoleic acid, conjugated diene and UV light absorption indicated that the influence of blackseed essential oil against oxidation of fats, especially in concentration of 0.06 g and 0.1 g of oil, increased by increase in temperature (Erkan *et al.*, 2012). In some cases, the essential oil antioxidant activity was less than extract and may not have a significant antioxidant activity compared to extract. As an example, the antioxidant activity of Iranian pennyroyal essential oil in vegetable oil during storage was investigated. Different concentrations of essential oil (0, 200, 400, 600, 800 and 1000 ppm) and BHT (200 ppm) were added to the sunflower oil emulsion in the presence of copper ions and were incubated in 60°C for 7 days. The amount of peroxide (PVs) and thiobarbituric acid reactive substances (TBARS) was determined every day for seven days. Additionally, the antioxidant capacity of essential oil was measured using β -carotene and DPPH methods. The results indicated that the findings of DPPH and β -carotene-linoleic acid method for *Mentha pulegium* extracts were comparable to the findings of BHT. In addition, in all incubation time points, the amount of PVs and TBARS for *M. pulegium* extracts were less than control sample. In this regard, water extract was more powerful than methanol extract. Essential oil didn't show a significant antioxidant effect. It seems that water extract of *M. pulegium* was a more powerful antioxidant and the essential oil of this plant had a weaker antioxidant activity comparing to extracts (Kamkar *et al.*, 2010). Some essential oils of spices (cumin, caraway, clove, thyme, rosemary and sage) and their main components were added to linoleic acid emulsion in aqueous media in order for investigating their antioxidant activity. The methods used for evaluating conjugated diene formation, thiobarbituric acid and linoleic acid oxidation included coupled oxidation of β -carotene. Investigated essential oils had powerful antioxidant effect and this effect was enhanced by increase in their concentration. In general, the effect of different essential oils on linoleic acid oxidation was provided in descending order as follows: caraway > sage > cumin > rosemary > thyme > clove. It showed that there was a relationship between the antioxidant activity and chemical composition of essential oils (Farag *et al.*, 1989). In order to study the oxidation behaviour, simple model system comprised of purified cottonseed oil and thyme essential oil and clove essential oil were designed. Three methods were used for

following the oxidation of cottonseed oil, which include coupled oxidation with β -carotene, the TBA test and hydroperoxide value. The results indicated that clove and thyme essential oils showed antioxidant activity in different concentrations and this effect was more powerful on clove essential oil than thyme essential oil. Sensory evaluation tests indicated that addition of thyme essential oil or clove essential oil to cottonseed oil in range of 50 to 1200 ppm didn't have a significant effect on cottonseed oil smell. Therefore, clove and thyme essential oils were suggested as natural antioxidants to prevent the oxidation of fats (Faraga *et al.*, 1989). In another study, the effect of essential oils of seven plants of thyme, clove, turmeric, sage, rosemary, cinnamon and oregano enriched by carvacrol on oxidative stability of corn oil in frying temperature was investigated. The test was done using PetroOxy instrument. Evaluating the effect of essential oil concentration (1500–5000 ppm) and temperature (150–180 °C) was used in induction of corn oil. Synthetic antioxidant of BHT was also used as a positive control. Induction periods observed from the accelerated oxidation test indicated that temperature increase, decreased the induction time in all samples and essential oils except oregano did not have a significant antioxidant effect on corn oil, which probably was related to their lower active compounds. Furthermore, it was observed that the antioxidant effect of oregano oil was higher than BHT (Inanc & Maskan, 2013). The essential oil extracted from six plants (*Monodoura tenuifolia*, *Lippia rugosa*, *Cymbopogon citratus*, *Lippia multiflora*, *Pimenta racemosa* and *Ocimum gratissimum*) was evaluated regarding the antioxidant activity. Therefore, they were determined using the conjugated autoxidizable triene (CAT) method, which was applied in aquatic emulsion of stripped tung oil. The essential oil of *P. racemos* had high amounts of chavicol (10.3%) and eugenol (54.5%) and showed powerful antioxidant activity and had the second rank after *O. gratissimum* in possessing high amounts of thymol (50.2%). The essential oil extracted from *M. tenuifolia* had a medium antioxidant activity. This essential oil contained high amounts of sesquiterpene alcohols like α -cadinol (20.5%) and its isomers, α -muurolol (14.7%) and germacrene D-4-ol (16.8%). The essential oils resulted from *L. multiflora* and *C. citratus* showed weak ability in protecting tung oil against oxidation. Finally, the essential oils containing phenol compounds, which had smaller amounts of sesquiterpene alcohols, showed the highest amount of CAT, which indicated that these compounds were the main determiners of antioxidant activity of these essential oil in water-oil emulsion (Tchobo *et al.*, 2013). In another study, the effect of ultraviolet irradiation (30 min), ultrasound treatment (30 kHz, 100 W, 30 s) and marzeh khuzistani essential oil (0.08%) on oxidation of canola oil was investigated. The analysis of GC-MS essential oil of marzeh khuzistani indicated that carvacrol (88.6%) was the major composition of essential oil. The amount of peroxide value, anisidine value, thiobarbituric acid, free fatty acids and induction period was measured in the oil and indicated that essential oils had antioxidant activity comparing to BHT in treated and untreated samples with ultrasound and UV. In addition, essential oil had the ability to decrease stable radical of DPPH with 50% incubation concentration (IC_{50}) $\mu\text{g} / \text{ml}$ 0.06 ± 29 . The results indicated that UV and ultrasound treatment increased the oil oxidation but essential oil could act as an antioxidant and increased stability of oil (Hashemi *et al.*, 2013).

Mayonnaise with diverse formulation was stored in 38 °C and oxidative stability and sensory effects of the produced sauce with various concentrations of Zenyan essential oil (0.015%, 0.03% and 0.045%) were compared to BHT and BHA. The quality parameters of mayonnaise indicated that all concentrations of essential oil had antioxidant

effect comparing to BHT and BHA. Adding essential oil in 0.045% had the highest stability during the storage. Essential oil was also able to decrease DPPH radicals with 50% inhibition concentration (IC_{50}) (Gavahian *et al.*, 2013). In another study, two concentrations of *Satureja bachtiarica* Bunge essential oil (0.03% and 0.06%) on oxidative stability of rapeseed oil during autoxidation and after radiating with UV for 30 min in 60°C was investigated. Essential oil contained thymol (30%), caryophyllene oxide (12%) and carvacrol (15%). Antioxidant activity of essential oil with BHT during oxidation was compared. UV rays caused oxidation in both samples with and without essential oil. This effect was stronger before adding essential oil and antioxidant. In addition, it was revealed that in samples treated by UV rays, the essential oil antioxidant activity was better than BHT (Tables 8.1 and 8.2). This essential oil could also decrease DPPH radicals with 50% inhibition concentration (IC_{50}) of $32.5 \pm 0.6 \mu\text{g/mL}$ (Hashemi *et al.*, 2011a). The effect of using 0.025%, 0.05% and 0.075% *Zataria multiflora* Boiss essential oil on oxidative stability of sunflower oil was evaluated and essential oil with BHA and BHT during storage was compared in 37°C and 47°C. The main compositions of essential oil included carvacrol (45.6%), *p*-cymene (18.1%) and thymol (16.3%). Measuring the amount of peroxide, anisidine and thiobarbituric in sunflower oil showed that all concentrations of essential oil had lower antioxidant effect comparing to BHT and BHA. Samples treated with essential oil concentration 0.075% had the highest stability during storage in both temperatures. Moreover, measuring the amount of totox, stabilisation factor, antioxidant activity and antioxidant power, approved the effectiveness of this oil as an antioxidant in sunflower oil (Table 8.3) (Hashemi *et al.*, 2011b). In another study, different concentrations (0.02%, 0.04%, 0.06% and 0.08%) of *Satureja khuzestanica* essential oil on oxidative stability of sunflower oil were investigated and compared to BHA during storage at 60°C. GC-MS of essential oil indicated that carvacrol (87.7%) was the main component of essential oil. Measuring peroxide value and anisidine value

Table 8.1 F-value and IP of rapeseed oil samples (Source: Hashemi *et al.*, 2011a).

Sample	IP(h)	F-value
Blank + UV	26.53 ^a	0.86 ⁱ
RO + EO – 0.03% + UV	30.01 ^d	0.97 ^l
UV + RO + EO – 0.3%	28.01 ^b	0.91 ^j
RO + EO – 0.06% + UV	31.21 ^c	1.01 ^k
UV + RO + EO – 0.06%	29.18 ^e	0.94 ^m
RO + BHT – 0.02% + UV	27.81 ^b	0.9 ^j
UV + RO + BHT – 0.02%	26.45 ^a	0.85 ⁱ
RO + EO – 0.03%	33.55 ^f	1.09 ⁿ
RO + EO – 0.06%	49.4 ^g	1.6 ^o
RO + BHT – 0.02%	37.44 ^h	1.21 ^p
Blank	30.86 ^c	–
Control	53.94 ^f	–

RO, rapeseed oil; EO, *S. bachtiarica* essential oil; IP, induction period; F, stabilisation factor. Means with the same lowercase letters are not significantly different at $P < 0.05$.

Table 8.2 Effect of EO, BHT and UV irradiation on anisidine value of rapeseed oil samples (Source: Hashemi *et al.*, 2011a).

Sample	0h	24h	48h	72h	96h	120h
Blank + UV	3.4 ± 0.08 ^a	13.36 ± 0.47 ^a	34.14 ± 1.32 ^a	67.22 ± 2.32 ^a	118.18 ± 3.23 ^a	165.12 ± 3.46 ^a
RO + EO 0.03% + UV	3.4 ± 0.08 ^a	10.06 ± 0.39 ^b	27.17 ± 1.11 ^b	61.16 ± 2.4 ^b	102.02 ± 2.9 ^b	149.33 ± 3.38 ^b
UV + RO - EO_0.3%	3.4 ± 0.08 ^a	12.94 ± 0.4 ^a	32.62 ± 1.4 ^a	68.34 ± 2.7 ^a	115.16 ± 3.11 ^a	160.3 ± 3.32 ^a
RO + EO_0.06% UV	3.4 ± 0.08 ^a	10.13 ± 0.31 ^b	27.16 ± 1.53 ^b	59.24 ± 1.97 ^b	99.13 ± 3.14 ^b	145.54 ± 3.51 ^b
UV + RO + EO_0.06%	3.4 ± 0.08 ^a	11.84 ± 0.43 ^a	30.63 ± 1.6 ^a	67.12 ± 2.44 ^a	113.14 ± 2.77 ^a	159.3 ± 3.14 ^a
BHT_0.02% UV RO	3.4 ± 0.08 ^a	12.06 ± 0.45 ^a	31.44 ± 1.05 ^a	68.53 ± 2.36 ^a	112.13 ± 3.04 ^a	158.7 ± 3.26 ^a
UV + RO + BHT_0.02%	3.4 ± 0.08 ^a	13.42 ± 0.53 ^a	33.23 ± 1.67 ^a	69.13 ± 1.82 ^a	114.92 ± 3.1 ^a	162.11 ± 3.18 ^a
RO + EO_0.03%	3.4 ± 0.08 ^a	6.4 ± 0.17 ^c	16.06 ± 0.91 ^c	44.36 ± 1.02 ^c	62.19 ± 1.22 ^c	101.29 ± 3.21 ^c
RO + EO_0.06%	3.4 ± 0.08 ^a	5.08 ± 0.09 ^d	11.12 ± 0.73 ^d	29.38 ± 0.89 ^d	55.16 ± 1.14 ^d	85.19 ± 2.78 ^d
RO + BHT_0.02%	3.4 ± 0.08 ^a	8.3 ± 0.44 ^e	22.6 ± 1.12 ^e	52.33 ± 2.07 ^e	80.07 ± 1.57 ^e	123.33 ± 3.24 ^e
Blank	3.4 ± 0.08 ^a	10.05 ± 0.19 ^b	28.17 ± 1.86 ^b	60.08 ± 1.66 ^b	98.46 ± 2.66 ^b	147.73 ± 3.41 ^b
Control	3.4 ± 0.08 ^a	4.2 ± 0.07 ^f	8.36 ± 0.11 ^f	19.08 ± 0.24 ^f	44.36 ± 1.09 ^f	73.12 ± 1.83 ^f

RO, rapeseed oil; EO, *S. bachtiarica* essential oil.Means with the same lowercase letters are not significantly different at $P < 0.05$.

Table 8.3 Comparison of the AA of BHT, BHA and EO in sunflower oil based on the peroxide value test during storage at 37 and 47 °C (Source: Hashemi *et al.*, 2011b).

Parameter	Temperature	BHA-0.02%	BHT-0.02%	EO-0.075%
IP (h)		240.48 ± 2.7 ^d	261.6 ± 2.5 ^l	288.48 ± 2.6 ^t
AA	37	3020 ± 7 ^a	3705 ± 8.4 ^b	1229 ± 5 ^c
F		1.6 ± 0.09 ^e	1.74 ± 0.1 ^f	1.92 ± 0.12 ^g
AOP		37.7 ± 0.23 ^h	42.6 ± 0.28 ^k	48 ± 0.27 ⁿ
IP (h)		205.2 ± 2.1 ^m	224.4 ± 2.3 ^o	249.36 ± 2.5 ^p
AA	47	3050 ± 7.4 ^a	3800 ± 8.6 ^b	1280 ± 5.2 ^c
F		1.61 ± 0.09 ^e	1.76 ± 0.11 ^f	1.96 ± 0.13 ^g
AOP		38 ± 0.24 ^h	43 ± 0.27 ^k	49 ± 0.3 ⁿ

EO, *Z. multiflora* essential oil; IP, induction period; AA, antioxidant activity; F, stabilisation factor; AOP, antioxidant power.

Means with the same lowercase letters are not significantly different at $P < 0.05$.

in sunflower oil indicated that all concentrations of essential oil had antioxidant effect comparing to BHA. The samples of oils treated with essential oil concentration 0.08% showed the highest stability during storage (Hashemi *et al.*, 2012). Different concentrations (0.025%, 0.05% and 0.075%) of *Carum copticum* essential oil on oxidative stability of sunflower oil was investigated and compared to BHA and BHT during the storage period at 37 °C and 47 °C. The main compositions of essential oil included thymol (50.07%), γ -terpinene (23.92%) and *p*-cymene (22.9%). Measuring the amount of peroxide, anisidine and thiobarbituric acid in sunflower oil, indicated that all concentrations of essential oil had antioxidant effect, comparing to BHT and BHA.

The samples in which, essential oil was added in 0.075%, had the highest stability during the storage in both temperatures. Therefore, the results indicated that the essential oil might be used as a natural antioxidant in edible oils (Hashemi *et al.*, 2011c). The effect of different essential oil on increased oxidative stability of peanut oil at 60 °C was investigated. Essential oil of *Origanum majorana*, *Acantholippia seriphioide* and *Tagetes filifolia* showed significant antioxidant activity in 0.02% level and after them *Rosmarinus officinalis* and *Eucalyptus cinerea* showed an average antioxidant activity (Yanishlieva & Marinova, 2001). In another study, after extracting essential oils from Dill seed, the essential oil was analysed by GC-MS and its chemical components were recognised. In order to investigate the antioxidant activity of essential oils, two tests of DPPH radical and β -carotene-linoleic acid assay were used. Moreover, its antioxidant behaviour in soybean oil was evaluated by measuring peroxide and thiobarbituric acid value and the results of these tests were compared to chemical antioxidants of BHT and BHA. The results indicated that D-carvone (36.09%), lymonen (19.89%), trans-dihydrocarvone (7.36%), cis-dihydrocarvone (6.59%) and thymol (6.5%) were the major components of essential oil. In DPPH test, the amount of EC_{50} in dill essential oil was calculated $2/57 \pm 1/52$ mg/ml, while this parameter for BHT was 0.038 ± 0.001 mg/ml. In β -carotene linoleic acid assay, the activity of essential oil in concentration of 7 mg/mL was equal to 77.29%, that antioxidant activity had proximity with BHT in 0.1 mg/mL. Antioxidant activity of dill essential oil in soybean oil at 0.6 mg/mL concentration level was almost equal to chemical antioxidant of BHA at 0.1 mg/mL concentration level

(Eyooghi *et al.*, 2009). Antioxidant activity of essential oils of thyme and its application in soybean oil as an alternative to synthetic antioxidants was investigated. In this investigation, essential oil of the plant was analysed using GC-MS and its major chemical components were recognised. Antioxidant activity of essential oil in different concentrations was investigated using two methods DPPH and β -carotene bleaching assay and was compared to BHT. Antioxidant activity of thyme essential oil was evaluated by measuring peroxide and thiobarbituric acid value in soybean oil (oven test). The results indicated that carvacrol (26.08%) and thymol (17.23%) were the major phenol components of essential oil. Thyme EC_{50} was measured 2.22 ± 0.04 mg/ml while this parameter was 0.58 ± 0.02 mg/ml for BHT. Antioxidant activity of essential oil in β -carotene bleaching assay was measured 72% in 0.45% level and was measured 81% for BHT in 0.01% level. In oven test, thyme at 0.1% concentration had an antioxidant effect equal to BHA at 0.02% concentration in soybean oil (Shahsavari *et al.*, 2008). The antioxidant activity of *Lavandula angustifolia* essential oil in soybean oil was investigated. The components of essential oil were recognised by GC-MS. The antiradical activity of essential oil was investigated using different methods and then the antioxidative activity of essential oil in soybean oil was studied using the oven test. Six major components of essential oil were linalool (27.89%), camphor (10.82%), 1,8-cineole (9.05%), linalool acetate (8.86%), borneol (7.29%) and α -terpineol. EC_{50} of *L. angustifolia* essential oil was calculated 35.54 ± 1.58 mg/mL. The maximum antioxidative activity belongs to 40 mg/mL concentration of essential oil. In $ABTS^{\cdot+}$ test, the maximum antioxidative activity of *L. angustifolia* essential oil belonged to 40 mg/mL concentration of essential oil. In β -carotene bleaching assay, the most anti-oxidative activity of *L. angustifolia* essential oil belonged to its 10 mg/mL concentration, which showed 54.4% inhibition. In oven test, the essential oil had the ability to prevent the primary and secondary products of oxidation in soybean oil at 0.8 mg/mL concentration level, which approximately equelled to BHA in 0.2 mg/mL concentration level (Tahanejad *et al.*, 2011). *Satureja* essential oil contains phenol compositions like carvacrol and thymol had high levels of antimicrobial and antioxidant properties. The phenol composition of *Satureja* essential oil was considered as a source of natural antioxidant compositions and the level of antioxidant activity of extracted compositions in rapeseed oil and anchovy fish oil was determined. Effects of antioxidants in following concentrations: 0.05%, 0.1%, 0.2%, 0.3% *Satureja* essential oil was compared to the control group and BHA (0.02%) artificial antioxidant; and was investigated by evaluating peroxide and thiobarbituric acid value and ransimat test in rapeseed oil and Anchovy fish oil. Carvacrol, γ -terpinene, α -terpinene and *p*-cymene were recognised as the main components of *Satureja* essential oil. The amount of all phenol compositions of *Satureja* essential oil was calculated 0.739 ± 0.279 mg Gallic acid/ml. Essential oil in 0.3% concentration showed the best antioxidant effect in preventing the increase in peroxide and thiobarbituric acid value. The results indicated that *Satureja* essential oil was a natural antioxidant and a proper alternative to BHA for stabilising and preserving the quality of fish and rapeseed oil (Amini *et al.*, 2015). The effect of antioxidant activity of different levels of *Foeniculum vulgare* essential oil including the concentrations 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 mg/mL in purified soybean oil was investigated at 90°C for four weeks in fixed seven days time intervals with oven test using peroxide, anisidine measurement indices and calculating totox index. In addition, the time of resistance against oxidation of samples was measured using the rancimat method. Then, the results from oven test and Rancimat method were compared to control group and BHA and BHT. Based on the results obtained from

this research, antioxidant effect increased as the essential oil concentration increased, but in the concentration range investigated there wasn't a linear direct relationship between treatments and their antioxidant activity in soybean oil. Both 0.5 and 0.6 mg/mL concentrations of *Foeniculum vulgare* essential oil had higher antioxidant activity comparing to BHA and BHT in soybean oil (Mazaheri Kalaroodi *et al.*, 2014).

8.4 Meat and Poultry Products

Meat products were sensitive to oxidation process and had a low stability toward this process. Oxidation process led to deterioration of tissue, odour, taste and colour in these products. Therefore, in order to control and decrease this process, antioxidants were used. Having antioxidant effect, essential oils could be useful in this regard. Some restrictions of using essential oils in meat products included unpleasant effects of some essential oils on odour and taste of these products, so not all essential oil could be used in meat products (Bozkurt, 2006; Djenane *et al.*, 2012; Ranucci *et al.*, 2014).

The antioxidant activity of α -tocopheryl acetate and oregano essential oil on ham and chicken breast sensitive to oxidation of fats during storage as frozen in -20°C for nine months was investigated. Eighty day-old chickens were randomly divided to four groups and were fed with basal diet including 30 α -tocopherylacetate mg kg^{-1} as the control or basal diet with 200 α -tocopheryl acetate mg kg^{-1} or basal diet with 100 or 50 mg oregano essential oil kg^{-1} for 38 days before slaughter. Oxidation of fat was evaluated by investigating the formation of malondialdehyde after 0 and 7 days in fridge after 1, 3, 6 and 9 months storage in frozen status. The results indicated that all treated diets had significant effects on oxidative stability of broilers. The diet enriched by oregano essential oil in 100 mg/kg level had a significant effect comparing to 50 mg/kg level and the control diet was more effective in decreased oxidation, but it was less effective than α -tocopheryl acetate (Botsoglou *et al.*, 2003). The effect of adding rosemary essential oil in various levels (150, 300 and 600 ppm) on protein oxidative stability of the frankfurters was studied. Frankfurters without essential oil were considered as control. The amount of carbonyls resulted from protein oxidation significantly enhanced in fridge during storage and this increase was significantly higher in control frankfurters comparing to those treated by 300 and 600 ppm essential oil. Rosemary essential oil in 300 ppm and 600 ppm successfully protected heme molecules against decomposition and prevented from increased nonheme iron in frankfurters during the storage in fridge. Colour change had a direct relationship with oxidation process because frankfurters with antioxidant (300 and 600 ppm) had less change in colour comparing to controls. Adding rosemary essential oil, by decreasing hardness, chewiness, gumminess, adhesiveness, increased tissue properties of frankfurters and controlled losing elasticity during storage in fridge (Estevez *et al.*, 2005). The effect of natural (*Thymbra spicata* essential oil and green tea extract) and BHT antioxidant on TBARS values and biogenic amine and quality (pH, colour and sensory attributes) of Turkish dry-fermented sausage during ripening was investigated. Adding antioxidant led to decreased TBARS values. It was revealed that the effect of natural antioxidant in decreasing the formation of TBARS was more than BHT. The power of antioxidants in decreasing the formation of putrescine was as follows:

green tea extract > green tea extract > *T. spicata* essential oil > *T. spicata* essential oil > BHT

Their average amount was 70.45 mg/kg, 76.05 mg/kg, 83.13 mg/kg, and 95.97, respectively. The highest concentration of tyramine belonged to control sausages without antioxidant, while the lowest amount belonged to those that respectively received 99.43 mg/kg and 64.31 mg/kg green tea extract. B, L, pH and total quality of sensory attributes didn't have a significant difference when adding green tea extract, *T. spicata* essential oil and green tea extract – *T. spicata* essential oil. This study points out that some natural antioxidants like essential oil were more effective than synthetic antioxidants, thus they could be used in meat products to increase quality and production of safe products (Bozkurt, 2006). Estevez and Cava (2006) studied the effect of increased concentration of rosemary essential oil (150, 300 and 600 ppm) on protein and fat oxidation and increased content of nonheme of frankfurters produced by Iberian pigs or White pigs during storage in fridge (+4°C/60 day). Frankfurters without essential oil were considered as control group. Iberian pigs were bred by free-range method and were fed in pasture, while white pigs were bred intensively (large - white × Landrace) and were fed with mixed diet. The obvious difference found between various types of frankfurters (Iberian versus white), was regarded the composition of fatty acid and tocopherols content, which was related to different foods of animals. The effect of adding rosemary essential oil on oxidative stability of frankfurters was related to the amount of added essential oil and frankfurters' attributes. Rosemary essential oil successfully restrained the progress of lipid and protein oxidation in Iberian pigs and the antioxidant effect was stronger in higher concentrations. In white pigs, 150 ppm rosemary essential oil had antioxidant effect and significantly decreased the production of protein and lipid oxidation products. Higher level of essential oil (300 and 600 ppm) generally didn't have any effect on lipid oxidation, while increased protein oxidation and release of iron from myoglobin. The presence of a certain amount of tocopherols in frankfurters could affect the activity of added essential oil, which resulted in antioxidant or prooxidant effects, although different compositions of fatty acid and oxidative status between frankfurters could bring about the same effects. The effect of pre-formulated mixture of commercial extract comprised of equal amounts of oregano essential oil and sweet chestnut wood extract, on oxidation status and the quality of pork was investigated. The results indicated that adding these compositions led to decrease lipid oxidation, improved odour and taste of the pork (Ranucci *et al.*, 2014). The effect of nutritional supplements resulted from essential oils (*Rosmarinus officinalis*) and artemisia (*Artemisia herbaalba*) on antioxidant status of muscles and oxidative stability of lamb was studied. Eighteen Barbarine lambs were divided into three groups and received oat hay and concentrates for 95 days. One group was without supplement, while two other groups received 400 mg/kg essential oils from rosemary or artemisia. Both essential oils had antioxidant attributes and their oral administration, improved the ability to revive and restrain muscle's free radicals comparing to the control group. However, essential oils supplement couldn't prevent lipid oxidation and didn't have any effect on colour stability of the meat kept in aerobic situation for seven days (Aouadi *et al.*, 2014). Nieto *et al.* (2013) studied the effect of two levels (0.4 and 0.05%) of rosemary essential oil, garlic, oregano on protein oxidation in park patties during storage in modified atmosphere (MAP: 70% O₂: 20% CO₂: 10% N₂) or under anaerobic situation at 4°C. Oxidative stability of meat protein was studied by evaluating the index of losing thiol groups after 9 days of storage, and formation of myosin cross-links by SDS-PAGE after 12 days of storage. Thiol protein groups were deteriorated during storage and were changed to

myosin disulfide cross-links. The results showed that essential oil of rosemary and oregano decreased the rate of deteriorating thiol groups and decreased the rate of myosin cross-link formation. On the contrary, garlic essential oil increased protein oxidation that was along with losing thiol groups and cross-link formation comparing to the control group. The antioxidant effect of cassia essential oil on deep fried beef during frying process was investigated. The results indicated antioxidant effect of cassia essential oil under this situation: 15 min at 150°C, 30 mL cassia oil/250 mL palm oil was optimum (Du and Li, 2008). The minced meat treated by essential oil of *Lavandula angustifolia* L. and *Mentha piperita* L. had the least TBARS (lipid oxidation). The main components of *L. angustifolia* were: (22.35%) linalool, (21.80%) linalyl acetate, (6.16%) trans-ocimene and (5.19%) 4-terpineol; and the main components of *M. piperita* included: (33.28%) menthol, (22.033%) menthone and (6.40%) menthyl acetate (Djenane *et al.*, 2012). The antioxidant activity of the meat treated by sage and oregano essential oil, while storing the meat was evaluated by these methods: thiobarbituric acid, DPPH and crocin assay. Minced samples of beef and pork were divided into three different groups: control (without antioxidant), oregano (oregano essential oil 3% w/w) and sage (sage essential oil 3% w/w). Then, the antioxidant activity of samples of each treatment stored in 4°C as raw or cooked (at 85°C for 30 min), after 1, 4, 8 and 12 days of storage was measured. The results indicated that essential oil treatments significantly decreased oxidation, while thermal treatment and storage duration was significantly influenced by antioxidant activity of meat. It seems that the role of antioxidants was more significant in cooked meat than raw meat and the quality of meat proteins was significantly influenced by antioxidant activity (Fasseas *et al.*, 2007). Milk protein-based edible films included mixed essential oil of oregano (w/v) 1.0%, pimento (w/v) 1.0%, or oregano pimento 1.0%, was used on beef to increase shelf-life during storage at 4°C. The meat treated with films were tested for biochemical analysis, periodically in seven days. Meat fat oxidation was evaluated by measuring thiobarbituric reactive substances (TBARS). Oregano-based films decreased fat oxidation in beef samples, while pimento based films led to highest antioxidant activity (Oussalal *et al.*, 2004). The effects of diet with oregano essential oil and α -tocopheryl acetate supplement on stability of raw and cooked turkey breast and thigh meat proportional to fat oxidation, during storage in fridge for nine days was tested. Thirty 12-week turkeys were divided to five groups and were fed basal diet including 30 mg kg⁻¹ oral α -tocopheryl acetate as control, or basal diet with 200 α -tocopheryl acetate mg kg⁻¹, or basal diet with 100 oregano essential oil mg kg⁻¹, or basal diet with 200 oregano oil mg kg⁻¹, or basal diet with 100 oregano oil mg kg⁻¹, and 100 α -tocopheryl acetate mg kg⁻¹ for four weeks before slaughter. Fat oxidation was investigated by measuring the amount of malondialdehyde in raw and cooked meat in 0, 3, 6 and 9 days of storage in fridge, using the third-order derivative spectrophotometric method. The results indicated that the treated diet significantly increased stability against fat oxidation in both raw and cooked turkey, comparing to control group. Oregano essential oil in 200 mg kg⁻¹ had a significant role in delaying fat oxidation, comparing to 100 mg kg⁻¹, equal to α -tocopheryl acetate in 200 mg kg⁻¹, but was less than oregano oil plus α -tocopheryl acetate in 100 mg kg⁻¹ for each of them; which in turn had a special prominence amongst all treated diets. In all treatments, thigh muscle comparing to breast muscle, was more susceptible to oxidation, although had a significant amount of α -tocopheryl acetate (Botsoglou *et al.*, 2003). The effect of oregano essential oil in Spanish fermented dry-cured sausages (salchichon) on

proteolysis, lipolysis and sensory attributes was evaluated. In order to evaluate decomposition intensity during sausage ripening process, the profile and fatty acid was measured. Adding oregano essential oil led to increased unsaturated fatty acid, but lipolysis was not significantly influenced. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SD-PAGE), which was used for qualifying proteolytic changes in sarcoplasmic and myofibrillar proteins during the process, had a similar pattern in all sausages. Oregano essential oil didn't have a significant effect on sensory attributes (Martín-Sánchez *et al.*, 2011).

8.5 Dairy Products

Butter as a dairy product, was susceptible to oxidation because of having high fat, therefore using preservative substances such as antioxidants could help its stability against oxidative processes. Essential oil as an antioxidant, which was used in many foods, besides having positive aspects like being natural, had some limitations like creating unpleasant flavour and taste. The effect of different essential oils (*Thymus haussknechtii* Velen and *Origanum acutidens*; native species of Turkey) on butter stability was investigated. These essential oils were added to butter in two concentrations of 0.1 wt% and 0.2 wt%. Antioxidant activity of essential oils was compared to control groups and samples containing BHT. All samples were stored in $4 \pm 1^\circ\text{C}$ for 90 days, and their peroxide values and thiobarbituric acid values, were compared. According to the results, the least amount of peroxide values and thiobarbituric acid values were found in samples containing BHT and 0.2% essential oils; however, peroxide values and thiobarbituric acid in control groups had the highest level in control samples. Approximately 0.2% level of essential oils showed more powerful antioxidant activity, which in most cases was equal to BHT. *T. haussknechtii* essential oil indicated more powerful antioxidant activity, comparing to *O. acutidens*. Sensory investigation of butter showed that samples containing 0.2% essential oil had less flavour scores comparing to those containing 0.1% essential oils. These results indicated that these essential oil could be used as an alternative to synthetic antioxidants in butter production (Dagdemiir *et al.*, 2009). In another study, cumin and thyme essential oil was used to prevent butter deterioration at room temperature. Butter oxidation and lipolysis was investigated by measuring thiobarbituric acid and peroxide value. Lipolytic activity and number of lipolytic bacteria were also measured. During butter storage, very small changes appeared in peroxide and thiobarbituric acid values, while acid value was gradually increased. Adding 200 ppm cumin and thyme essential oils to butter led to slight increase in acid value. Thyme and cumin essential oils had more anti-hydrolytic effect and better preservation effect than BHT (Farag *et al.*, 1990). Antioxidant activities of *Satureja cilicica* essential oil was investigated in butter. Essential oil with 0.5% and 1.0% and 2.0% was added to butter as antioxidant and was evaluated during storage in 4 and 20°C for 60 days. Peroxide value, titratable acidity, pH and total lactic acid bacteria were investigated as a scale to evaluate antioxidant activity of essential oil in twentieth, fortieth and sixtieth days of storage. The essential oil of *S. cilicica* showed more powerful antioxidant activity. Antioxidant activity of essential oils increased as the essential oils concentration increased. Furthermore, peroxide value, titratable acidity, pH and number of viable lactic acid bacteria were compared to control samples. Essential oil of *S. cilicica* can be used as an antioxidant and a flavouring factor in butter (Ozkan *et al.*, 2007).

8.6 Conclusions

Nowadays, majority of consumers of food materials prefer natural compositions. Essential oils are amongst the natural compositions that can have several useful properties, especially antioxidant activity in foods and they can protect foods against oxidation. Phenol compositions of essential oils are more active in this area and can increase shelf-life of foods. Some limitations of using essential oils in food like undesirable odour and taste can be eliminated by selecting proper essential oils. Essential oils have numerous advantages like being natural that can restrict undesirable effects on consumers.

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9

Mode of Antioxidant Action of Essential Oils

Riccardo Amorati¹ and Mario C. Foti²

¹ Department of Chemistry 'G. Ciamician', University of Bologna, Italy

² Istituto di Chimica Biomolecolare del CNR, Italy

9.1 Introduction

There is a growing need for innovative food preservation systems. Reduction of food waste is recognised as a priority target by both FAO (2016) and the European Commission (2016). Antioxidants represent an important class of food preservatives because they are able to slow down the oxidation of unsaturated lipids (LH) contained in food, preventing therefore the development of rancidity (Schaich, 2005). Atmospheric oxygen (together with microorganisms) is the main oxidant species responsible for the decay of the organoleptic qualities of food. The reaction involved (called lipid autoxidation or peroxidation) is mediated by free radicals, which catalyse the formation of lipid hydroperoxides (LOOH) with a radical-chain mechanism (Schaich, 2005). Butylated hydroxyanisole (BHA, E320), butylated hydroxytoluene (BHT, E321), propyl gallate (PG, E310) and *tert*-butylhydroquinone (TBHQ, E319) are synthetic antioxidants commonly added to food to inhibit lipid peroxidation and the consequent rancidification. The impact of these petroleum-derived antioxidants on human health is still under debate and generally natural alternatives are increasingly preferred (Williams *et al.*, 1999; Carocho *et al.*, 2014).

In the last years, essential oils (EOs) have been actively investigated to replace synthetic antioxidants. Essential oils consist of a complex mixture of low-boiling compounds which can be extracted from aromatic plants by steam distillation and other techniques. Generally, these mixtures contain mono- and sesqui-terpenes, oxygenated terpenes, *phenols*, and other compounds. In most cases, the antioxidant activity of essential oils can be easily explained by the presence of phenols in their composition. Phenols are well-known antioxidants able to trap the chain-carrying lipid peroxy radicals, LOO[•] responsible for lipid oxidation (Foti, 2007). However, during the past decades it has been discovered that other simple components of EOs may contribute, although less efficiently, in the trapping process of LOO[•] radicals. The EOs that contain significant amounts of these hydrocarbon molecules manifest antioxidant activity in model systems as well as in real foods not attributable to

phenols. The addition of EOs in food can be beneficial because, in addition to their antioxidant abilities, EOs often have antimicrobial, antimycotic and flavouring properties (Amorati *et al.*, 2013).

A research performed on the 'Web Of Science' (Thomson Reuters) in November 2015 afforded 1164 articles for the combined keywords 'essential oil, antioxidant and food', with the number of publications rapidly increasing in the last few years (see Figure 9.1). This testifies that the research of EOs with antioxidant activity and the study of the structure-activity relationship are therefore an important field of the current research on antioxidants. However, a clear understanding of the molecular mechanism involved in these processes, despite the large number of published papers, has not been reached yet. This is because most of the chemical methods used to assess the antioxidant activity are often inappropriate to the target and investigations on real samples of food are instead scarce (Amorati *et al.*, 2013). As will be shown in Section 9.3, many popular assays used to estimate the antioxidant activity (AA) are not related to lipid peroxidation and thus they have little predictive value. So, it is not unusual to find that some EOs manifest AA for a few researchers and not for others (Amorati *et al.*, 2013). The use of EOs in food has also potential drawbacks that cannot be ignored. Their intense flavour may be unwanted and their volatility may cause loss during storage or cooking (Amorati *et al.*, 2013). Further, certain EO components are highly oxidisable so that the composition of EOs may change during storage for the formation of unknown compounds (Turek & Stintzing, 2013). Several essential oil constituents are toxic for humans (Adams & Taylor, 2009) and topical exposure to a few air-oxidised EO components (*e.g.*, linalool) may cause the development of skin allergies (Christensson *et al.*, 2010). In truth, the fact that EOs are 'natural' does not automatically mean that they are safe! In this context, some researchers underline the requirement of specific studies in order to determine the EO acceptable daily intake (Carocho *et al.*, 2014; Adams & Taylor, 2009; Burdock & Carabin, 2009).

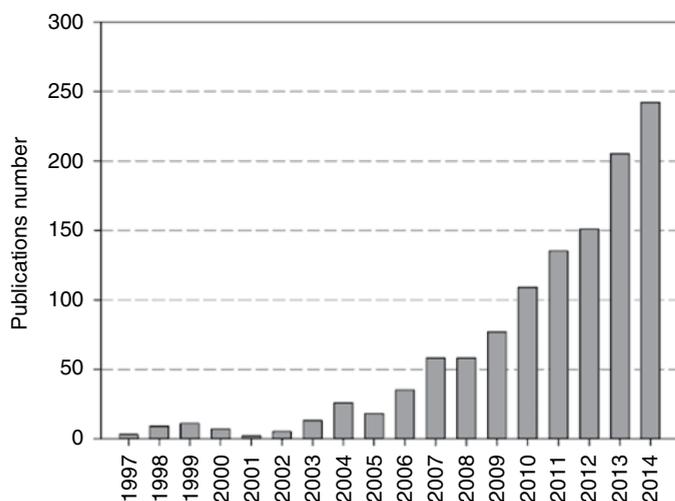


Figure 9.1 Increasing number of publications concerning EOs with antioxidant activities and food in the last 18 years.

9.2 Lipid Oxidation and Antioxidant Activity of Chemical Compounds

The oxidation process to which we refer is the oxidation of organic material (in most cases unsaturated lipids, L-H) caused by molecular oxygen in its triplet state (the common oxygen we breathe) at room temperature with formation of hydroperoxides (LOOH) as *primary* oxidation products, Reaction 9.1 (Schaich *et al.*, 2005).



Despite the apparent innocuity of Reaction 9.1, lipid oxidation (also called lipid *autoxidation* or *peroxidation*) is one of the major reasons of degradation of food during prolonged storage, with deterioration of texture, flavour, nutrition, colour and vitamin content over time (Schaich, 2014). Prolonged peroxidation goes far beyond the formation of hydroperoxides. Lipid chains are broken down into small oxygenated compounds in a process of increasing complexity which gives off the typical off-odours and off-flavours of stale vegetable oils. To some extent, this process resembles the combustion process of organic matter which, however, requires higher temperatures. Lipid peroxidation transfers damage to proteins through its oxidation products (*i.e.*, radicals, hydroperoxides, epoxides, reactive carbonyl compounds), which cause the formation of lipid adducts, scission, denaturation, aggregation and crosslinking of proteins (Schaich, 2014). Such damage reduces the nutritional value of food well 'beyond production of rancid flavours and odours' (Schaich, 2014). This aspect is particularly relevant when it is evaluated in terms of: (i) pollution generated by food production on the one hand, and (ii) increasing world population on the other hand, which, according to the United Nations Department of Economic and Social Affairs, will reach *ca.* 10 billion in 2055 (2016). The need of inexpensive, safe and effective antioxidants for food is therefore urgent. The use of synthetic phenolic antioxidants (such as BHA, BHT, PG, *etc.*; see above) could in the future be limited because of health concerns, and thus essential oils with antioxidant *and* antimicrobial activities could have an important role in the prevention of the oxidation processes and consequences mentioned above.

The appraisal and choice of effective antioxidants, able to reduce the rate of lipid peroxidation and the damage to proteins, are subjected to the knowledge of the mechanism of oxidation. In the advanced stages, lipid peroxidation is very destructive of the lipid chains and the involved (stepwise) mechanisms are not entirely understood yet (Denisov & Afanasev, 2005). On the other hand, the mechanism of *primary* oxidation which leads to the formation of hydroperoxides, as represented in Reaction 9.1, is instead well-known and has been tested many times during these past 50 years with coherent results (Denisov & Afanasev, 2005). It is evident that any chemicals able to retard the formation of hydroperoxides (by intervening in the primary mechanism of oxidation) are also able to retard the formation of *secondary* oxidation products, including off-flavours. Furthermore, these substances can prevent or limit the modification of proteins (Schaich, 2014).

Several decades of research on oxidative degradation of hydrocarbons in air have demonstrated that Reaction 9.1 occurs through a radical-chain mechanism characterised by three distinct phases: initiation, propagation, and termination (Denisov & Afanasev, 2005). In the first stages of the chain-reaction, a few initiating radicals (In^{*})

are generated. The presence of UV-light, ionising radiations, transition metals, lipoxygenases, hydroperoxides and so on, facilitates the formation of these species (Foti, 2007). Afterwards, these In^{\bullet} radicals abstract fairly quickly (see below) an H-atom from the allylic positions of unsaturated lipids (see Scheme 9.1). The carbon radicals so formed, L^{\bullet} , react *rapidly* with molecular oxygen to give peroxy radicals, LOO^{\bullet} . The reaction is often diffusion-controlled providing the concentration of dissolved O_2 is $\geq 0.1 \text{ mM}$ ($P_{\text{O}_2} \approx 0.1 - 1 \text{ atm}$) (Denisov & Afanasev, 2005). Peroxy radicals (LOO^{\bullet}) propagate oxidation ($\text{LOO}^{\bullet} + \text{L-H} \rightarrow \text{LOOH} + \text{L}^{\bullet}$) to the entire bulk of material until they are destroyed by other LOO^{\bullet} radicals (termination), see Scheme 9.1. Free radicals exert therefore a catalytic action on Reaction 9.1 because oxygen cannot react with lipids L-H (rule of spin conservation).

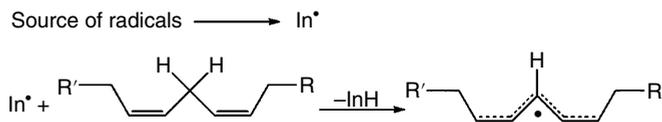
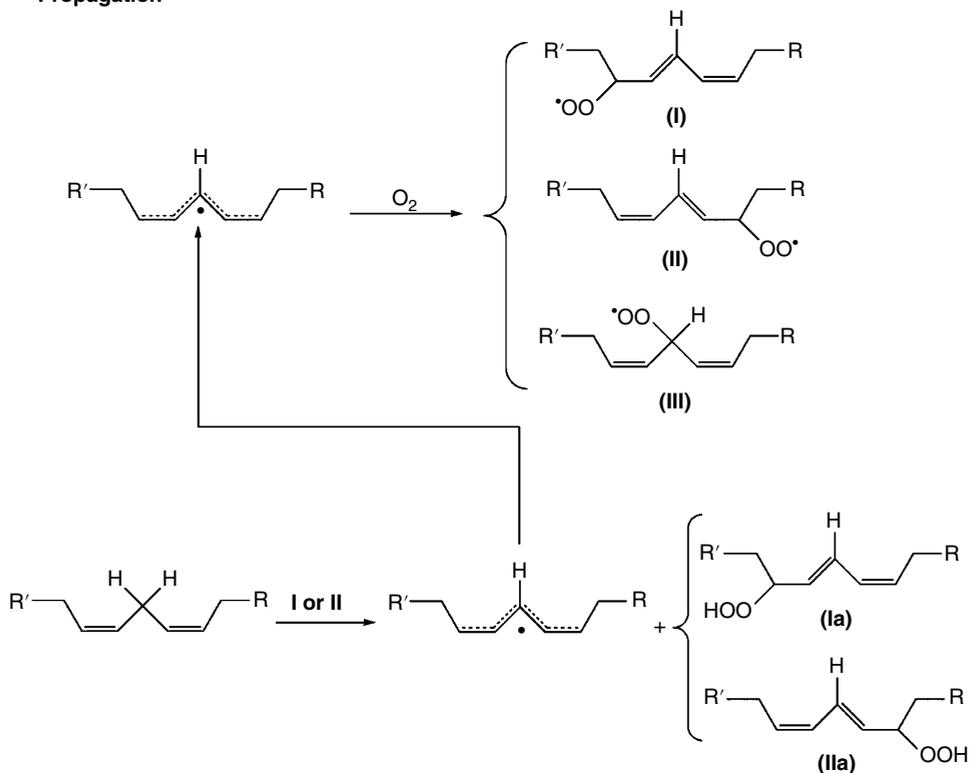
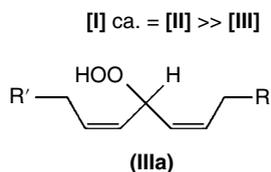
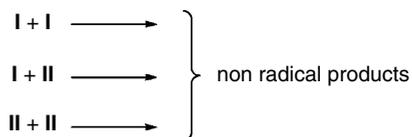
Peroxidation of unsaturated hydrocarbons commences at the allylic positions due to the proximity of the π -system which decreases the dissociation enthalpy of the allylic C-H bond by stabilising the L^{\bullet} radical (Foti, 2007). Allylic C-H bonds are therefore excellent H-atom donors. The rate constants $k(\text{LOO}^{\bullet} + \text{allylic C-H})$ are in fact comparatively large. For instance, oleic acid methyl ester (18:1) reacts with LOO^{\bullet} with a rate constant $k = 1.1 \text{ M}^{-1} \text{ s}^{-1}$; linoleic acid methyl ester (18:2) with $k = 60 \text{ M}^{-1} \text{ s}^{-1}$; linolenic acid methyl ester (18:3) with $k = 120 \text{ M}^{-1} \text{ s}^{-1}$; and arachidonic acid methyl ester (20:4) with $k = 180 \text{ M}^{-1} \text{ s}^{-1}$ (Schaich, 2005). For comparison, the rate constant for H-abstraction by LOO^{\bullet} from saturated hydrocarbons is $\leq 0.001 \text{ M}^{-1} \text{ s}^{-1}$ (Foti, 2007; Denisov & Afanasev, 2005).

In Scheme 9.1, peroxidation of a 1,4-diene hydrocarbon with *cis* geometry (e.g., linoleic acid) is shown. Removal of an H-atom from the *bis*-allylic position generates a pentadienyl radical that is stabilised by resonance. This radical may react with O_2 at either terminal carbon atoms, thus forming the peroxy radicals I or II (Tallman *et al.*, 2004). Most oxygen consumed by the autoxidation process is used to form the hydroperoxides Ia and IIa (Porter & Wujek, 1984). On the other hand, the *bis*-allylic hydroperoxide IIIa is not formed because the corresponding *bis*-allylic peroxy radical III (which does form rapidly) undergoes a rapid β -fragmentation ($k \sim 10^6 \text{ s}^{-1}$) that regenerates the pentadienyl radical (Tallman *et al.*, 2004). In the presence of large concentrations of α -tocopherol (0.05–1.8 M) the radical III, however, is rapidly reduced to IIIa which becomes the main hydroperoxide formed in the oxidation process (Tallman *et al.*, 2004).

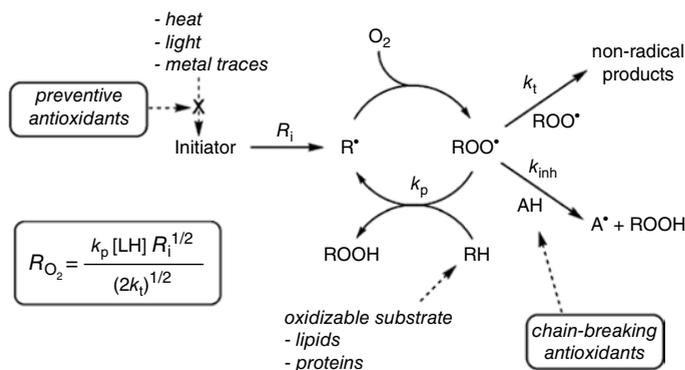
Another important modification caused by the oxidation regards the double bonds in Ia and IIa which are in conjugated position and with a different geometry (*trans-cis* and *cis-trans*) than in the original lipid, see Scheme 9.1 (Tallman *et al.*, 2004). In general, free radicals (particularly thiyl radicals) catalyse *cis-trans* isomerisation in lipids (Chatgialiloglu & Ferreri, 2005). This process seems to be detrimental to human health since *trans* unsaturated fatty acids are associated with a higher risk of coronary heart disease.

To reduce the rate of lipid peroxidation, it is necessary to act upon one of the three distinct phases of chain-reactions analysed above (Denisov & Afanasev, 2005). The kinetic rate law of free peroxidation (Reaction 9.1) clearly shows the effects of these processes, see Scheme 9.2 (Foti, 2007; Denisov & Afanasev, 2005).

The equation shown in Scheme 9.2 can conveniently be used to recognise the actions necessary to reduce the rate of peroxidation. In principle, peroxidation could be prevented by eliminating oxygen from food. However, oxygen is, on the one hand, useful to prevent formation of anaerobic bacteria and, on the other hand, its elimination is very

Initiation

Propagation

Termination


Scheme 9.1 Mechanism of peroxidation of hydrocarbons with bis-allylic (e.g., linoleic acid) C-H bonds. The overall process is a radical-chain reaction in which the LOO^\bullet radical is the chain-carrier. The typical three phases of chain reactions, that is, initiation, propagation and termination, are shown. The hydroperoxide IIIa does not form because its precursor radical III quickly regenerates the pentadienyl radical by oxygen loss.



Scheme 9.2 Schematic representation of the peroxidation of a substrate. The equation that appears in the scheme is the kinetic rate-law for free (*i.e.*, not inhibited) peroxidation. R_{O_2} is the rate of O_2 uptake; k_p the rate constant of propagation; k_t the rate constant of termination, and finally R_i the rate of initiation. The points of attack of preventive and chain-breaking antioxidants on the chain-reaction are shown: preventive antioxidants block the formation of radical initiators; chain-breaking antioxidants remove ROO^* radicals from the system limiting therefore the propagation process.

difficult and costly. Thus, antioxidants represent the only viable route to prevent or retard oxidation.

The retardation of peroxidation can effectively be achieved by using preventive or chain-breaking antioxidants (Foti, 2007; Denisov & Afanasev, 2005; Ingold & Pratt, 2014). preventive antioxidants which act by reducing the initial formation of free radicals (*i.e.*, the initiation rate). Hydroperoxide or H_2O_2 reducers, metal-ion chelators and 1O_2 quenchers (see Scheme 9.2) are examples of preventive antioxidants. Chain-breaking antioxidants AH act instead by scavenging LOO^* radicals, Reaction 9.2, with a consequent reduction of the rate of propagation, see Scheme 9.2 (Foti, 2007; Denisov & Afanasev, 2005; Ingold & Pratt, 2014). Generally, the radical A^* produced by Reaction 9.2 is not sufficiently reactive to sustain peroxidation because most chain-breaking antioxidants AH are aromatic (*e.g.*, phenols) and the (oxygen- or nitrogen-centred) A^* is strongly stabilised by resonance (Ingold & Pratt, 2014). Good chain-breaking antioxidants are in fact phenols, aromatic amines, pyridinols, pyrimidinols and ascorbate (Foti, 2007; Ingold & Pratt, 2014; Pratt *et al.*, 2001).

The antioxidant ability of AH is determined by the thermochemistry of Reaction 9.2. Generally, antioxidants with A-H bond dissociation enthalpies lower by several kilocalories per moles than those of $LOO-H$ (≈ 88 kcal/mol) are effective antioxidants (Ingold & Pratt, 2014).



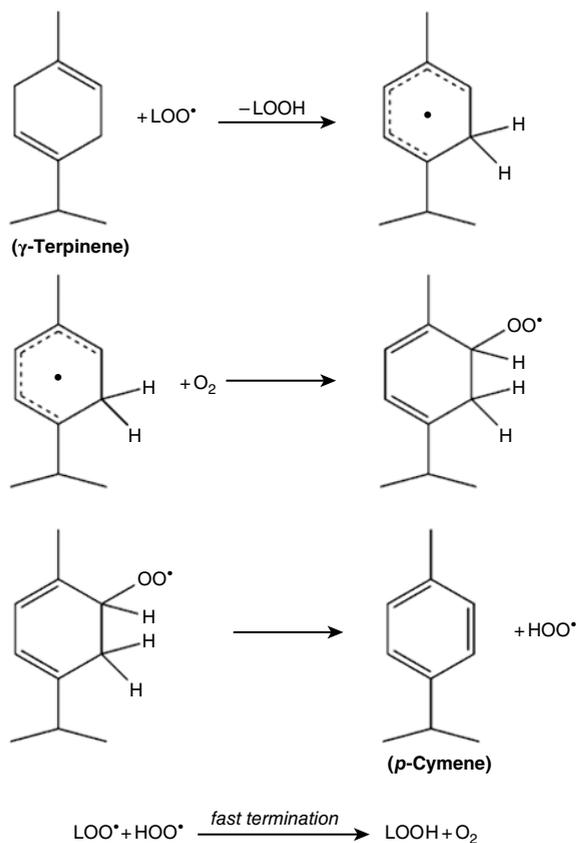
A-H bonds should not be too weak, as in this case a direct reaction with oxygen is feasible ($AH + O_2 \rightarrow A^* + HOO^*$), which has pro-oxidant rather than antioxidant effect. In kinetic terms, AH is effective when $k_{inh} \gg k_p$ (see Scheme 9.2). α -Tocopherol, which is considered one of the best antioxidants in living organisms, has a rate constant k_{inh} of $3.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Burton *et al.*, 1985). Rate constants k_{inh} for typical chain-breaking phenolic antioxidants are reported in Table 9.1 (Burton *et al.*, 1985; Pedrielli *et al.*, 2001;

Table 9.1 Rates of reactions of selected (poly)phenols with peroxy radicals, from autoxidation studies.

Phenol	$k_{inh}/M^{-1}s^{-1}$	Solvent	References
α -tocopherol	3.2×10^6	PhCl, 30 °C	Burton <i>et al.</i> (1985)
BHT	1.4×10^4	PhCl, 30 °C	Burton <i>et al.</i> (1985)
epicatechin	4.2×10^5	PhCl, 50 °C	Pedrielli <i>et al.</i> (2001)
	1.7×10^4	<i>t</i> -BuOH, 50 °C	Pedrielli <i>et al.</i> (2001)
quercetin	5.0×10^5	PhCl, 30 °C	Amorati <i>et al.</i> (2013)
	2.1×10^4	<i>t</i> -BuOH, 50 °C	Pedrielli <i>et al.</i> (2001)
hydroxytyrosol	8.0×10^5	PhCl, 30 °C	Amorati <i>et al.</i> (2013)
	3.0×10^4	MeCN, 30 °C	Amorati <i>et al.</i> (2013)
Caffeic acid	2.9×10^5	PhCl, 1% MeOH, 30 °C	Amorati <i>et al.</i> (2006)
Caffeic acid phenetyl ester	6.8×10^5	PhCl, 30 °C	Spatafora <i>et al.</i> (2013)
	1.3×10^4	MeCN, 30 °C	Spatafora <i>et al.</i> (2013)
Resveratrol	1.8×10^5	PhCl, 30 °C	Tanini <i>et al.</i> (2015)
	1.0×10^4	MeCN, 30 °C	Tanini <i>et al.</i> (2015)
3,4-Dihydroxybenzoic acid	6.5×10^4	PhCl, 1% MeOH, 30 °C	Amorati <i>et al.</i> (2006)
Epigallocatechin gallate	1.3×10^4	SDS micelles, pH7.4, 37 °C	Zhou <i>et al.</i> (2005)
Magnolol	6.1×10^4	PhCl, 30 °C	Amorati <i>et al.</i> (2015)
	6.0×10^3	MeCN, 30 °C	Amorati <i>et al.</i> (2015)
Honokiol	3.8×10^4	PhCl, 30 °C	Amorati <i>et al.</i> (2015)
	9.5×10^3	MeCN, 30 °C	Amorati <i>et al.</i> (2015)

Amorati *et al.*, 2013; Amorati *et al.*, 2006; Spatafora *et al.*, 2013; Tanini *et al.*, 2015; Zhou *et al.*, 2005; Amorati *et al.*, 2005.

As mentioned in the introduction, a few EOs free of phenols have antioxidant properties. Their mechanism of action, however, does not usually fall into any of the two main groups mentioned above. γ -Terpinene and EOs that contain this monoterpene have the ability to retard the peroxidation of linoleic acid (Ruberto & Baratta, 2000; Foti & Ingold, 2003). Concentrations of γ -terpinene as small as 1 mM reduce the rate of linoleic acid oxidation by a factor of 10 (Foti & Ingold, 2003). These antioxidant properties arise from a peculiar mechanism of co-oxidation of γ -terpinene, see Scheme 9.3. The linoleylperoxy radicals abstract an H-atom from the bis-allylic CH_2 's present in γ -terpinene to yield a terpenoyl radical that reacts rapidly with oxygen. In turn, the terpenoylperoxy radical formed abstracts *intramolecularly* a H-atom to give *p*-cymene and $HOO\cdot$. The release of $HOO\cdot$ causes an acceleration in the chain-termination due to the cross-reaction between $HOO\cdot$ and $LOO\cdot$ radicals that occurs at diffusion-controlled rate ($k = 3 \times 10^9 M^{-1}s^{-1}$ at 50 °C in cyclohexane), see Scheme 9.3, while the rate constant of termination for the secondary linoleylperoxy radicals ($LOO\cdot + LOO\cdot$) is much slower $2k_t = 8.8 \times 10^6 M^{-1}s^{-1}$ at 30 °C (Foti & Ingold, 2003). Consequently, essential oils containing γ -terpinene could be used as promising



Scheme 9.3 Mechanism of transformation of γ -terpinene in p -cymene in the presence of linoleic acid. The transformation that occurs during the co-oxidation of linoleic acid causes the release of HOO^\bullet radicals which cross-terminate quite rapidly the (linoleic acid) chain-carrier, LOO^\bullet .

antioxidants at least when their aroma is not in contrast with the food organoleptic characteristics (Ruberto & Baratta, 2000; Foti & Ingold, 2003).

9.3 Methods for Determining the Antioxidant Properties of Chemicals

Lipid oxidation can be prevented or retarded by the use of suitable antioxidants. The principles that govern the antioxidant properties are essentially known (see above) and this has guided the isolation of hundreds of antioxidants from natural sources and the synthesis of new compounds with maximised antioxidant properties (Ingold & Pratt, 2014; Pratt *et al.*, 2001; Burton *et al.*, 1985). Experimental methods for the evaluation of the antioxidant potential of natural or synthetic compounds are however necessary. Several methods exist but not all of them are sound. A few of them will be briefly described here; further details can be found in recent reviews on this topic (Foti, 2015; Amorati & Valgimigli, 2015).

9.3.1 Autoxidation of a Substrate

The best method to measure the antioxidant properties of chemicals is based on the peroxidation of an oxidisable substrate (linoleic acid, styrene, cumene, *etc.*) under controlled conditions, before and after the addition of antioxidants. The system mimics spontaneous peroxidations that occur in natural or man-made organic products. The rate of peroxidation of the substrate can be measured by monitoring oxygen uptake or formation of hydroperoxides. In a closed system, oxygen consumption can be monitored by means of: (i) a pressure transducer (Viglianisi *et al.*, 2014); (ii) an oxygen electrode (Amorati *et al.*, 2006); (iii) an oxygen-sensitive fluorescence probe (Hay *et al.*, 2006). In the case of hydroperoxides their formation can be monitored by (i) iodometric assay (peroxide value) (Foti, 2015); (ii) UV spectroscopy (Foti & Ruberto, 2001); and (iii) HPLC analysis (Amorati *et al.*, 2016). All the measurement techniques have pros and cons and their choice is usually determined by convenience reasons. The profile of peroxidation rates with and without antioxidants gives a quantitative evaluation of the antioxidant efficacy, as shown in Figure 9.2. Most of the rate constants for Reaction 9.2 have been determined with this technique.

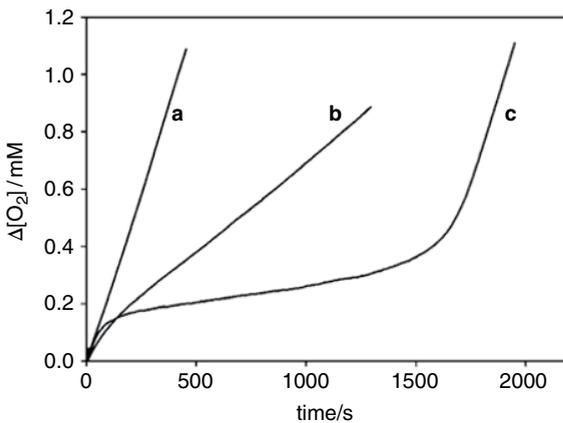
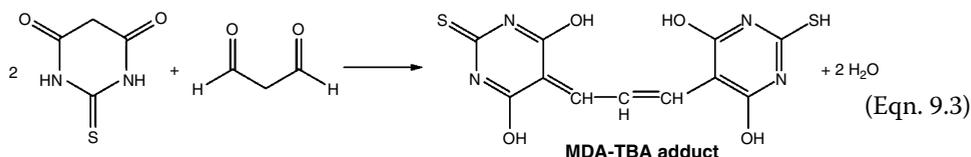


Figure 9.2 Example of typical oxygen consumption plots measured during the autoxidation of an organic substrate initiated by an azoinitiator in the absence of inhibitors (a) and in the presence of a weak (b) or of a strong (c) antioxidant.

9.3.2 TBARS (Thiobarbituric Acid Reactive Species)

Malondialdehyde (MDA) is a secondary product formed by the peroxidation of unsaturated lipids. This reactive carbonyl species is potentially mutagenic (Hartman, 1983). The coloured adduct that MDA forms with thiobarbituric acid (TBA), at relatively high temperatures (90–100 °C) under acid conditions, (Reaction 9.3), is correlated to the rate of peroxidation (Ohkawa *et al.*, 1979; Yagi, 1998). The MDA-TBA adduct is coloured in pink and can be determined colourimetrically ($\lambda \sim 532$ nm) or fluorometrically

(excitation λ at 532 nm and emission at 553 nm) (Sigma-Aldrich, 2014; Cayman, 2016). The presence of efficient antioxidants in the system determines a slower formation of lipid hydroperoxides and thus a lower concentration of MDA and MDA-TBA. The MDA determination can be done in several materials subject to peroxidation. Commercially available assay kits allow for the determination of MDA in human plasma, serum and urine (Cayman, 2016). Tissue homogenates, cell lysates and biologic samples in general (such as foods) can be analysed with TBARS assay, too (Cayman, 2016). The specificity of this assay has however been questioned since TBA can also react with compounds other than MDA (Amorati & Valgimigli, 2015).



9.3.3 The Rancimat Test

The Rancimat assay consists in the determination of volatile, secondary reaction products formed by spontaneous peroxidation of oils or fats (Viuda-Martos *et al.*, 2010). Oxidation is triggered by a stream of hot air (90–120 °C). Volatile oxidation compounds, transported by the air stream and absorbed in a vessel containing deionised water, generate in the solution an electrical conductivity that increases over time. The lag time until these ionising compounds are formed is called induction period. This resistance to the appearance of oxidation products is due to the antioxidant action and concentration of chemicals present in the sample. The induction time therefore characterises the oxidation stability of oils and fats.

9.3.4 ORAC Assay

The oxygen radical absorbance capacity (ORAC) is a test used to evaluate the antioxidant capacities of samples. This technique is based on the oxidative degradation of fluorescein caused by peroxy radicals ROO^{\bullet} generated in aqueous solution by azo-initiators, R-N=N-R (e.g., 2,2'-azobis(2-amidino-propane dihydrochloride)). In the presence of antioxidants, this degradation is retarded because the antioxidants neutralise ROO^{\bullet} , Reaction 9.2. The fluorescent intensity at 520 nm (excitation at 480 nm) of fluorescein decreases as the oxidative degradation by ROO^{\bullet} proceeds. The area of the decay curves (fluorescence intensity versus time) is recorded both in the absence and in the presence of antioxidant. These two areas are related to the efficiency of the antioxidant. Usually, the results are expressed in terms of Trolox-equivalents (Trolox is an α -tocopherol analogue), that is, the quantity of Trolox that provides the same protection. The main limitations of this technique are the absence of an oxidisable substrate, the fact that antioxidant activity is measured by the area under the curve (AUC) method, and the aqueous solvent that makes unpractical the analysis of compounds with limited water solubility (Amorati & Valgimigli, 2015).

9.3.5 DPPH Test

The DPPH test is a popular test for determining the antioxidant properties of synthetic and natural compounds (or plant extracts). This test is based on the colour variation

from purple to pale yellow that occurs during the reaction of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) with antioxidants, Reactions 9.4 and 9.5. The DPPH[•] radical is in fact intensely coloured in purple while its reduction product DPPH-H is yellowish.



The antioxidant properties of the compounds examined with this assay are expressed in terms of EC₅₀, that is, the concentration of AH that quenches (after a fixed time) 50% of the initial DPPH[•]. This parameter implies that compounds with a high antiradical potential have low EC₅₀'s. The validity of this test has recently been criticised (Foti, 2015) because the EC₅₀ parameter is inappropriate to express the antioxidant potential of chemicals. Further, essential oils free of phenols react with DPPH[•] slowly and with complex kinetics. For instance, DPPH[•] reacts with γ -terpinene with a rate constant of 0.00156 M⁻¹s⁻¹ and with autocatalytic kinetics (Foti *et al.*, 2008).

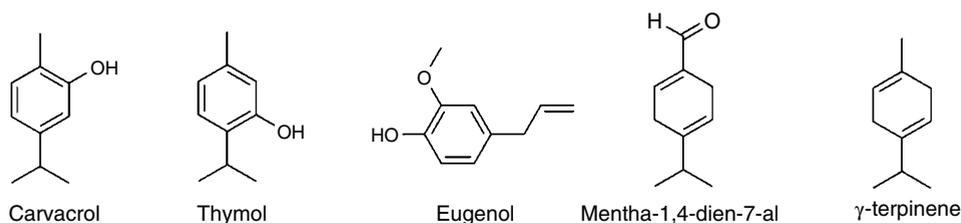
9.4 Antioxidant Activity of Essential Oils

In this section the antioxidant properties of EOs and their components are reviewed. As mentioned in the previous section, the most reliable data are those obtained from autoxidation studies including the Rancimat assay, which provides reliable data for a large number of EOs. The antioxidant activity of innovative formulations, such as active packaging and nano or micro particles containing EOs, are also examined because they are at the cutting edge of methods for improving food preservation.

9.4.1 Essential Oils Components

The antioxidant activity of EOs depends on the presence of components able to quench peroxy radicals. The most relevant components that impart antioxidant activity to an EO are those having a phenolic moiety (such as carvacrol, thymol and eugenol) and those possessing the cyclohexadienyl structure (such as γ -terpinene and mentha-1,4-dien-7-al), as reported in Scheme 9.4.

Yanishlieva *et al.* (1999) reported that the autoxidation of tocopherol-stripped triacylglycerols of lard (Figure 9.3A) and sunflower oil (Figure 9.3B) is inhibited by thymol and carvacrol (see Scheme 9.4), which are largely present in the EOs of thyme and oregano (about 50–70% by weight) (Yanishliev *et al.*, 1999).



Scheme 9.4 Phenolic and non-phenolic constituents of EOs with antioxidant properties.

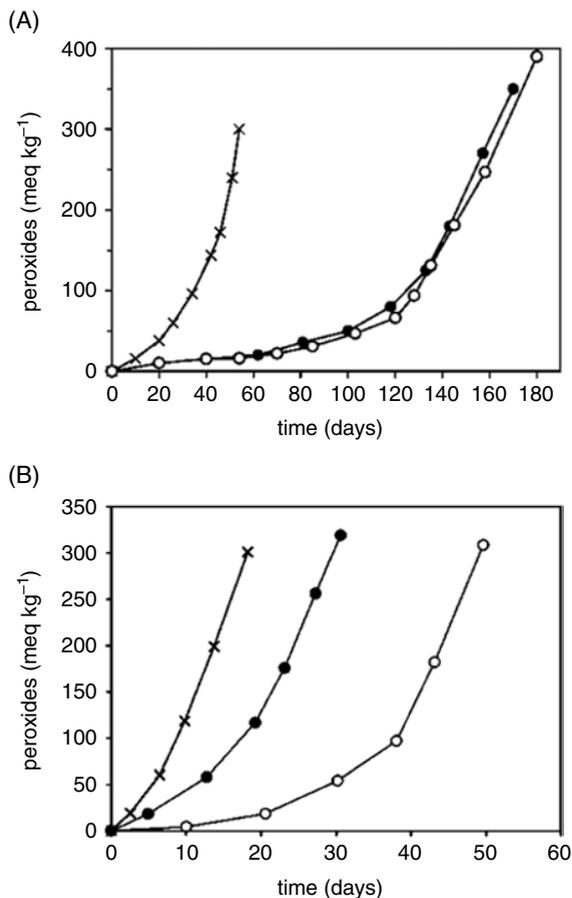
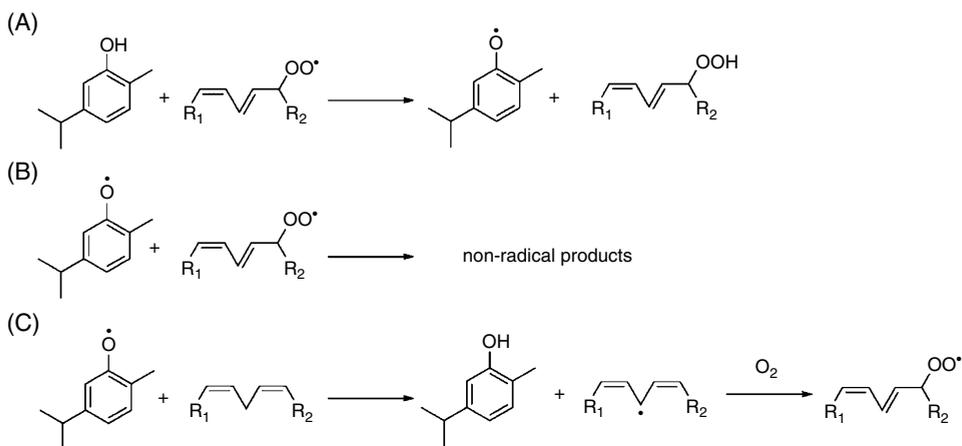


Figure 9.3 Formation of peroxides during spontaneous oxidation of triacylglycerols from lard (A) or from sunflower oil (B) at 22°C in the absence (x) and in the presence of 0.05% of carvacrol (●) and thymol (○). (Source: From Yanishlieva *et al.*, 1999).

The antioxidant activity was measured by following the formation of peroxides during spontaneous air-oxidation of the two lipids at room temperature. The concentration of peroxides was determined by iodometric titration of aliquots taken from the lipids (Figure 9.3). In the absence of inhibitors, the concentration of peroxides quickly increased, reaching the value of 300 mEq/kg after 50 days in the case of lard, or after 20 days in the case of sunflower oil. The fast spontaneous oxidation of the sunflower oil is due to its high unsaturation degree. In the presence of 0.05% carvacrol or thymol, the increase in the concentration of peroxides in the lard was delayed by about 120 days, relative to samples lacking these antioxidants, while in the case of the more oxidisable sunflower oil the inhibition was less evident and the induction time was shorter (see Figure 9.3B). Carvacrol and thymol showed in the lard the same antioxidant effect while in the sunflower oil, carvacrol was much less protecting than thymol. Yanishlieva *et al.* explained this behaviour by proposing that the phenoxyl radical of carvacrol might



Scheme 9.5 Carvacrol generates a phenoxyl radical after H-abstraction by LOO^{\bullet} , reaction A. This phenoxyl radical can quench another LOO^{\bullet} radical, reaction B, terminating therefore two oxidation chains (antioxidant action) or it can abstract an H-atom from the bis-allylic positions of the polyunsaturated lipids, reaction C. This last reaction produces another LOO^{\bullet} which propagates oxidation.

react with the bis-allylic CH_2s of the polyunsaturated fatty acids (see Scheme 9.5). This reaction would cause a propagation of the oxidative chain and therefore a decrease in the antioxidant effect of carvacrol. In the case of thymol, the greater steric hindrance of its phenolic group would reduce the reactivity of the radical toward polyunsaturated lipids (Yanishliev *et al.*, 1999).

The data reported in Figure 9.3 were obtained at room temperature. The authors noticed that when the temperature was increased to 100°C (the typical temperature adopted to accelerate the stability tests), the antioxidant abilities of carvacrol and thymol were very small, presumably because of their volatility (Yanishliev *et al.*, 1999). Mastelic *et al.* (2008) (by using the Rancimat test at 100°C) reported that carvacrol, thymol and eugenol at concentrations of 0.02% w/w increased the induction period in the oxidation of lard only by a factor of 1.37, 1.32, 1.39, respectively. For comparison, the synthetic antioxidant BHT (2,6-di-*tert*-butyl-4-methylphenol), which is less volatile, under the same condition increased the induction period by a factor of 3.55 (Mastelic *et al.*, 2008). The Rancimat test was also used by Loizzo *et al.* (2015) to study the protecting activity of several essential oil components over the autoxidation of tocopherol-stripped corn oil at 121.6°C (Loizzo *et al.*, 2015). At the maximum concentration, ca. 10% v/v, the only EO components that showed antioxidant activity were thymol, eugenol, and γ -terpinene, see Figure 9.4 (Loizzo *et al.*, 2015). Surprisingly, in these experiments eugenol was as active as the synthetic antioxidant BHT. On the other hand, *p*-cymene and linalool had negligible antioxidant activity, whereas β -caryophyllene and *trans*-cinnamaldehyde showed a marked pro-oxidant activity, see Figure 9.4.

The data shown in this brief paragraph demonstrate that only a limited number of EOs' components have antioxidant capabilities. These antioxidant compounds possess phenolic and cyclohexadiene moieties.

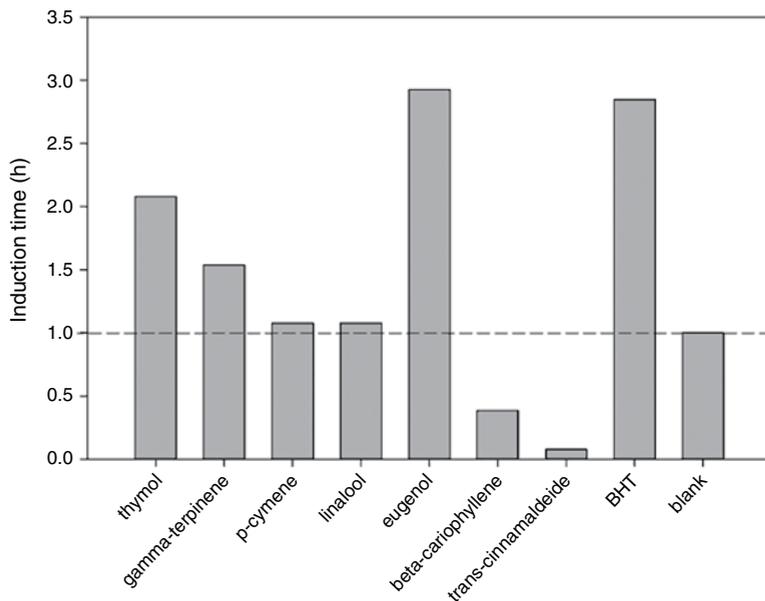


Figure 9.4 Antioxidant effect of some EO components compared to reference antioxidants, measured by the Rancimat test in corn oil at 121.6°C. (Source: From Loizzo *et al.*, 2015).

9.4.2 Antioxidant Activity of Essential Oils

Essential oils containing a high percentage of phenolic components or γ -terpinene (see previous paragraph) usually possess antioxidant activity. Loizzo *et al.* (2015) assessed by the Rancimat test (oxidation of tocopherol-stripped corn oil at 121.6°C) the antioxidant properties of EOs extracted from several aromatic plants (ginger, cumin, cinnamon, clove, lavender, grapefruit, lemon, anise star, rosemary, sage, bay, fennel, orange, basil, dill, pine needle, black pepper, lemongrass, bog myrtle, thyme, bergamot, peppermint, Spanish marjoram) and found that only the EOs of cinnamon, clove, thymus and cumin had antioxidant effects. These EOs had substantial amounts of antioxidant components. Cinnamon and clove EOs contained for instance 44% and 84% of eugenol, respectively; thymus EO 28% of thymol, cumin EO 24% of *p*-mentha-1,4-dien-7-al and 13% of γ -terpinene (see Scheme 9.4) (Loizzo *et al.*, 2015). The only exception was the bay EO that, despite the large presence of eugenol (43%), showed no antioxidant activity with the Rancimat test. It is likely that the antioxidant activity of eugenol activity was thwarted by the fast oxidation of other components, such as methyl eugenol (Amorati *et al.*, 2015). Turan reported that the onset of the oxidation of canola oil, evaluated by the Rancimat assay (oxidation of purified triacylglycerols from canola oil, 80°C) or the UV-vis detection of conjugated dienes, was delayed by thyme EO at concentrations of 500–2000 ppm. No effect was instead detected with sage, rosemary and bay EOs. The protective effects exerted by the EO of thyme were however lower than those of BHT and BHA (butylated hydroxyanisole) (Turan, 2014). Viuda-Martos used various techniques to assess the antioxidant

activities of thyme, oregano, clove, sage and rosemary EOs. With the Rancimat method (oxidation of unpurified lard, 120 °C, antioxidant concentration 50 g/L) only the first three EOs showed antioxidant activity, though smaller than that of BHT (Viuda-Martos *et al.*, 2010). These results were confirmed by the TBARS test in which the EO of clove had a better performance than BHT (Viuda-Martos *et al.*, 2010). Politeo *et al.* (2006) studied several EOs (clove, coriander, basil, mint, black pepper, laurel, marjoram, everlast, nutmeg, fennel, cinnamon, sage EOs) by measuring the oxidation delay of unpurified lard at 110 °C with the Rancimat apparatus to discover that only the EO of clove had antioxidant activity. Surprisingly, the EO of basil containing 11.6% of eugenol showed in these experiments no antioxidant activity (Politeo *et al.*, 2006). Kulisic *et al.* (2005) evaluated the effect of EOs from oregano, thymus and savory on the peroxidation of unpurified lard at 100 °C by using the Rancimat test (Kulisic *et al.*, 2005). They found that only the EOs containing significant amounts of phenolic components were able to retard the oxidation of lard. The separate contributions of the hydrocarbon (no phenols) and oxygenated fractions (with phenols) of these EOs were assessed. As expected, the hydrocarbon fraction of these EOs was ineffective or had pro-oxidant activity (Kulisic *et al.*, 2005). The antioxidant effects obtained with the Rancimat test for several EOs are collected in Table 9.2.

Table 9.2 Antioxidant activity of essential oil evaluated with the Rancimat assay.

Essential oil	Main components	Activity	References
Anise star (<i>Illicium verum</i>)	Anethole (62.3%), estragole (7.8%),	Pro-oxidant	Loizzo <i>et al.</i> (2015)
Basil (<i>Ocimum basilicum</i>)	Linalool (55.6%), α -bergamotene (7.4%),	None	Loizzo <i>et al.</i> (2015)
Bay (<i>Pimenta racemosa</i>)	Eugenol (43.2%), methyl eugenol (10.7%)	None	Loizzo <i>et al.</i> (2015)
Bergamot (<i>Citrus bergamia</i>)	Limonene (38.4%), linalyl acetate (28.2%), γ -terpinene (6.8%),	None	Loizzo <i>et al.</i> (2015)
Black pepper (<i>Piper nigrum</i>)	β -Caryophyllene (11.5%), limonene (10.8%),	Pro-oxidant	Loizzo <i>et al.</i> (2015)
Bog myrtle (<i>Myrica gale</i>)	β -Myrcene (18.1%), limonene (10.7%),	None	Loizzo <i>et al.</i> (2015)
Cinnamon (<i>Cinnamomum zeylanicum</i>)	Eugenol (43.5%), (E)-cinnamaldehyde (28.9%)	Antioxidant	Loizzo <i>et al.</i> (2015)
Clove (<i>Eugenia caryophyllata</i>)	Eugenol (83.8%)	Antioxidant	Loizzo <i>et al.</i> (2015)
Coriander (<i>Coriandrum sativum</i> L.)	Linalool (92%)	None	Politeo <i>et al.</i> (2006)
Cumin (<i>Cuminum cyminum</i>)	p-Mentha-1,4-dien-7-al (23.9%), cumin aldehyde (15.4%), γ -terpinene (13.1%),	Antioxidant	Loizzo <i>et al.</i> (2015)

(Continued)

Table 9.2 (Continued)

Essential oil	Main components	Activity	References
Dill (<i>Anethum graveolens</i>)	Carvone (43.5%), limonene (39.8%)	None	Loizzo <i>et al.</i> (2015)
Everlast (<i>Helichrysum italicum</i>)	α -Cedrene (18.3 %), α -Pinene (11.3 %) 2-Methylcyclohexyl pentanoate (10.5)	None	Politeo <i>et al.</i> (2006)
Fennel (<i>Foeniculum vulgare</i>)	Anethole (58.7%), estragole (8.1%),	Pro-oxidant	Loizzo <i>et al.</i> (2015)
Ginger (<i>Zingiber officinale</i>)	Zingiberene (23.6%), β -bisabolene (11.1%), β -sesquiphellandrene (10.3%),	Pro-oxidant	Loizzo <i>et al.</i> (2015)
Grapefruit (<i>Citrus paradisi</i>)	Limonene (88.7%)	None	Loizzo <i>et al.</i> (2015)
Bay laurel (<i>Laurus nobilis</i>)	1,8-Cineole (55.2), α -terpinyl acetate (12.4)	None	Turan (2014)
Lavender (<i>Lavandula angustifolia</i>)	Linalool (24.6%), linalyl acetate (14.7%), terpinen-4-ol (11.8%),	None	Loizzo <i>et al.</i> (2015)
Lemon (<i>Citrus limonum</i>)	Limonene (56.7%), γ -terpinene (12.3%)	None	Loizzo <i>et al.</i> (2015)
Lemongrass (<i>Cymbopogon flexuosus</i>)	Geranial (43.2%),	Pro-oxidant	Loizzo <i>et al.</i> (2015)
Marjoram (<i>Marjorana hortensis</i> Moench.)	Terpinen-4-ol (40.8 %), γ -terpinene (16.3%), α -terpinene (11.0%),	None	Politeo <i>et al.</i> (2006)
Nutmeg (<i>Myristica fragrans</i>)	Sabinene (25.4 %), α -pinene (15.8 %), myristicine (14.8 %), β -pinene (13.4)	None	Politeo <i>et al.</i> (2006)
Orange (<i>Citrus sinensis</i>)	Limonene (89.7%)	None	Loizzo <i>et al.</i> (2015)
Oregano (<i>Origanum vulgare</i>),	Thymol (35.0%), carvacrol (32.0%), γ -terpinene (10.5 %);	Antioxidant	Viuda-Martos <i>et al.</i> (2010)
Peppermint (<i>Mentha piperita</i>)	Menthol (52.4%), menthone (13.6%)	None	Loizzo <i>et al.</i> (2015)
Pine needle (<i>Pinus sylvestris</i>)	α -Pinene (22.9%), δ -3-carene (12.5%),	None	Loizzo <i>et al.</i> (2015)
Rosemary (<i>Rosmarinus officinalis</i>)	1,8-Cineole (29.1%), camphor (10.5%), borneol 12.3 (%)	None	Loizzo <i>et al.</i> (2015)
Sage (<i>Salvia officinalis</i>)	1,8-Cineole (32.5%), α -thujone (13.1%)	Pro-oxidant	Loizzo <i>et al.</i> (2015)
Spanish marjoram (<i>Thymus mastichina</i>)	1,8-Cineole (45.6%), limonene (9.8%)	Pro-oxidant	Loizzo <i>et al.</i> (2015)
Thyme (<i>Thymus vulgaris</i>)	Thymol (28.3%), m-cymene (16.9%)	Antioxidant	Loizzo <i>et al.</i> (2015)

9.4.3 Antioxidant Activity of Films, Edible Coatings and Nanomaterials Containing EOs

Inclusion of EOs in *nano-* or *micro-particles* may bring several advantages such as a better solubility in water, a better dispersion in food matrices and a reduction of their volatility. The antioxidant activity of these materials was evaluated and some examples are reported hereinafter. Recently, a protein extracted from maize (zein) was used to encapsulate thyme EO into particles with a diameter of 0.8–4 μm . The antioxidant effect of this material was evaluated by the Rancimat method, studying the oxidation of refined olive oil triacylglycerols at 100 and 120°C. Zein particles without EO were devoid of antioxidant activity. On the other hand, zein particles containing thyme EO showed the same antioxidant effect of free EO. This is because the nanoparticles released pretty quickly EO into the lipid phase. The activity of this EO was, however, considerably smaller than that of BHT and α -tocopherol (Bilenler, 2010). Likewise, the radical trapping activities, evaluated with the DPPH test, of soybean phospholipid liposomes loaded with clove EO or pure eugenol were superimposable to that of free eugenol (Sebaaly, 2015).

The recent interest for *active packaging* and *edible coatings*, used to improve food preservation, has suggested the preparation of novel materials containing EOs (Bonilla, 2012). Oregano EO and green tea extracts were incorporated in an ethylene–vinyl alcohol copolymer (EVOH) to obtain polymer films with antioxidant and antimicrobial properties, which might be used for active packaging of food. The radical trapping ability of the antioxidants released in a food-simulant medium were evaluated by the DPPH test. The results showed that the oregano EO was less effective than the green tea extract in quenching DPPH \cdot (Muriel-Galet *et al.*, 2015). Similarly, carvacrol and thymol were incorporated into polypropylene films and their radical scavenging activity toward DPPH \cdot was evaluated after these antioxidants were released in the medium (Ramos *et al.*, 2014).

There are many examples of studies on the antioxidant activity of *edible coating* in which EOs are incorporated. Thyme EO was for instance incorporated in quince seed mucilage films at concentrations $\leq 2.0\%$, and the radical trapping ability after their dissolution in water was assessed by the DPPH \cdot test. The quince seed mucilage alone had a limited activity, whereas the films loaded with thyme EO had a significantly larger DPPH \cdot -bleaching activity (Jouki *et al.*, 2014). Chitosan-based edible films incorporating *Zataria multiflora* Boiss EO (containing 65% of thymol), or grape seed extracts were prepared, and the antiradical activity was measured by the DPPH \cdot assay after dissolution in water. All films showed DPPH \cdot bleaching activity but the largest activity was observed with the films containing grape seed extracts (Moradi *et al.*, 2012). Atares *et al.* prepared sodium caseinate edible films incorporating cinnamon or ginger essential oils and tested their antioxidant ability by measuring the peroxide values in samples of sunflower oil covered by these films. The authors found that the sunflower oil was protected from oxidation because of the oxygen barrier created by the caseinate film whereas the incorporated EOs had had little, if any, influence (Atarés *et al.*, 2010). Examples of EOs incorporated into fish gelatine films can be found in the literature (Tongnuanchan *et al.*, 2013; Teixeira *et al.*, 2014; Pires *et al.*, 2013).

9.5 Antioxidant Activity of EOs in Real Food Samples

Most of the studies on the antioxidant activity of EOs and their pure components are performed in model systems, such as seed oils or lard. These systems are much simpler than real food where the existence of hydrophilic and lipophilic phases, the presence of metals or endogenous antioxidants, which could promote or inhibit peroxidation, and the occurrence of bacterial growth (which can determine a massive deterioration of food) often make the results of the 'tests' unreliable. Therefore, it is extremely important to evaluate *in real food* the antioxidant effect that EOs may have before deciding on their possible use. The compatibility of the aroma of essential oils with the organoleptic characteristics of food is also very important. Below, a few studies on the antioxidant activity of EOs in food are reported. These works are divided on how essential oils are employed: (a) mixed with foodstuff; (b) included in the packaging, or (c) as ingredients of edible coatings. The largest antioxidant effects are found when EOs are directly added to food and contain significant amounts of phenolics. In fact, incorporation of EOs in packaging or coating often gives contradictory results.

9.5.1 EOs Directly Added to Food

9.5.1.1 Fish

The deterioration of carp fillet after treatment with a thymol and carvacrol mixture, and with electrolysed NaCl solution (releasing free Cl_2) was investigated. The antioxidant activity was assessed by measuring the peroxide value and the TBARS of the samples. Results showed that the two EO phenols reduced the formation of both hydroperoxides and TBARS. The combined treatment with chlorine and EOs strongly reduced lipid peroxidation and prolonged from 4 to 16 days the shelf-life (Mahmoud *et al.*, 2006). Oregano EO rich in thymol, carvacrol and γ -terpiene, in combination with a modified atmosphere packaging (40% CO_2 , 30% O_2 and 30% N_2) was investigated to preserve salted sea bream fillets stored at 4°C. The antioxidant effect was determined by TBARS while the shelf-life and the quality retention characteristics were evaluated by sensory evaluation and release of volatile amines. The results showed that oregano EO reduced the TBARS value and was able to neutralise the pro-oxidant effect of the modified atmosphere. It also reduced the formation of volatile amines and increased the shelf-life from 16 days for untreated fish fillets to 33 days. The authors reported that oregano EO 'yielded a distinct but pleasant flavour' (Goulas & Kontominas, 2007). The effect of thyme EO and modified atmosphere packaging (5% O_2 , 50% CO_2 , 45% N_2) on the quality of swordfish fillets stored at 4°C was assessed by measuring TBARS, volatile amines, bacterial count and sensory evaluation. Addition of thyme EO (0.1 % v/w) improved oxidative stability of all samples particularly of those stored under modified atmosphere. On the basis of the analysed parameters, the shelf-life of swordfish fillets increased from about 6 to 12 days (Kykkidou *et al.*, 2009).

9.5.1.2 Meat

The effect of oregano EO on the oxidation and quality retention of pork meat during storage (at 6°C) was assessed by TBARS and sensory evaluation of raw and cooked meat. Bacterial count and protein degradation were also measured. Results showed that oregano EO had a strong antioxidant effect because it completely suppressed

malondialdehyde formation. On the other hand, the authors found only a little effect on the bacterial growth probably because of the low concentration of EO. Oregano EO extended the shelf-life of pork meat from 8 to 12 days (Michalczyk *et al.*, 2015). Basil EO was added to beef burgers, and the lipid peroxidation during 12 days of storage at 4°C was measured by the TBARS assay. The treatment with basil EO did not cause any effect on the oxidation of meat, in line with the lack of antioxidant components in basil EO (see Table 9.2). Surprisingly, even the addition of BHT did not modify significantly the rate of formation of TBARS (Sharafati-Chaleshtori *et al.*, 2015).

The effect of the addition of rosemary EO (150-600 ppm) on the peroxidation of pig frankfurters was studied by Estevez and Cama by measuring the TBARS value. As reported in Table 9.2, rosemary is devoid of antioxidant components; on the other hand, frankfurters already contained 0.05% of sodium ascorbate (a strong antioxidant) and other food additives (sodium nitrite and phosphates). The overall effect of EO addition was small, and depended on the kind of meat used to prepare frankfurters. In the case of meat from Iberian pigs, the authors observed a small reduction of TBARS with increasing concentrations of rosemary EO while in the case of white pigs the EO (at a concentration of 600 ppm) had pro-oxidant effect (Estevez & Cava, 2006). The antioxidant activity of sage and rosemary EOs (BHT as standard) on pork liver paté was investigated by TBARS and the change of the lipid profile. Since the investigated EOs do not contain antioxidant components (see Table 9.2), the paté was supplemented with 0.05% sodium ascorbate. The results showed that these EOs had minor effects on the disappearance of polyunsaturated fatty acids, though they significantly reduced TBARS formation as well as the formation of lipid-derived volatile compounds (such as hexanal). BHT had little influence on these parameters (Estevez *et al.*, 2007). The antioxidant effect of oregano and sage EOs on raw or cooked pork and beef meat was assessed by TBARS during storage at 4°C. The authors reported that in the presence of both EOs, the TBARS formation was drastically reduced. The antioxidant effect of oregano EO was stronger than that of sage EO. However, the authors did not comment on the antibacterial effects that these EOs could have despite the fact that bacterial contamination could be the cause of the TBARS formation. Since the essential oil of sage does not contain chemicals with antioxidant properties, the effects of this EO could well be due to reduction of bacterial spoilage (Fasseas *et al.*, 2008).

9.5.1.3 Oil

The changes of organoleptic characteristics of extra-virgin olive oil during storage with and without the essential oils of different varieties of oregano were assessed by sensory evaluation. Oregano EOs reduced the development of rancid flavour as an effect of their antioxidant activity (Asensio *et al.*, 2012). The results of the studies on the antioxidant effects of EOs directly added to food are summarised in Table 9.3.

9.5.2 EOs in Modified Atmosphere Packaging

This preservation technique consists of a packaging containing a reservoir of EO. The EO vapours in contact with the surface of food exert a protective effect. EOs can be placed in the packaging envelope or adsorbed at the bottom of the container. The only examples reported so far regard EOs employed to reduce browning and maintain the food texture of fruits and vegetables. In these cases the antioxidant action of EOs is only indirect

Table 9.3 Examples of the use of EOs or their components as antioxidants in food. The assays employed to evaluate the antioxidant properties of the added EOs are indicated in parenthesis.^a

Food group	food	Essential oil or components	Comment	References
Fish	Carp fillet	Thymol, carvacrol	Antioxidant effect (TBARS and peroxide value)	Mahmoud <i>et al.</i> (2006)
	Sea bream fillet	Oregano	Antioxidant effect (TBARS)	Goulas <i>et al.</i> (2006)
	Swordfish fillet	Thyme	Antioxidant effect (TBARS)	Kykkidou <i>et al.</i> (2009)
Meat	Pork shoulder	Oregano	Antioxidant effect (TBARS)	Michalczyk <i>et al.</i> (2005)
	Beef burger	Basil	No effect (TBARS)	Sharafati-Chaleshtori <i>et al.</i> (2015)
	Pork frankfurters	Rosemary	Weak antioxidant or prooxidant effect, depending on the meat origin (TBARS, hexanal)	Estevez <i>et al.</i> (2006)
	Liver paté	Sage, rosemary	No effect on PUFA degradation, antioxidant effect on TBARS	Estevez <i>et al.</i> (2007)
	Bovine and porcine meat	Sage, oregano	Antioxidant effect (TBARS)	Fasseas <i>et al.</i> (2008)
Oil	Olive oil	Oregano	Reduced rancid flavour during prolonged storage	Asensio <i>et al.</i> (2012)

^a TBARS = thiobarbituric acid reacting species; PUFA = polyunsaturated fatty acids.

because it is due to inhibitory effects on the enzyme tyrosinase. Two active papers containing cinnamon essential oil (main components: cinnamaldehyde and eugenol) were investigated as a method to prolong the shelf-life of mushrooms. Organoleptic characteristics of mushroom samples put in macroperforated PET trays covered with the active paper were monitored for nine days. The radical trapping activity of the active papers was estimated by dipping the paper in a methanolic solution of the DPPH[•] radical and by measuring the bleaching of the radical. The release of volatile components from the active paper was assessed by HSPME–GC–MS during the nine days. The results showed that the active paper reduced weight loss and browning of mushrooms essentially associated with the activity of tyrosinase (Echegoyen & Nerin, 2015). Several natural compounds (carvacrol, anethole, cinnamaldehyde, perillaldehyde, linalool, and p-cymene, *etc.*) were tested for their effectiveness in reducing the decay of blueberries and increasing their content in phenols. The EO components were placed in a small beaker inside the fruit container at 10°C. All tested compounds were able to reduce fruit decay without any relationship with the intrinsic antioxidant activity of the EO component, presumably as effect of tyrosinase inhibition (Wang *et al.*, 2008). Similar experiments were also performed with grapes (Valero *et al.*, 2006) and strawberries (Wang *et al.*, 2007).

9.5.3 Edible Coatings

Edible coatings are thin layers of proteins, lipids or polysaccharides that can be applied on foods to preserve their organoleptic characteristics and to control food contamination. These matrices may incorporate active ingredients, such as EOs or their components, yields bioactive coatings that have been tested with real food. The results obtained so far, however, are contradictory and indicate the need for further investigations. For instance, edible films produced with milk proteins and containing oregano (1.0% w/v) or pimento (1.0% w/v) EOs were applied on beef muscle slices stored at 4°C. This treatment had little effect on bacterial growth; further, the presence of essential oils in the coating did not improve the protection against lipid oxidation (Oussalah *et al.*, 2004). Sodium alginate coatings containing horsemint EO were studied as a method to improve the stability of bighead carp fillets during storage at 4°C. Oxidation was assessed by measuring the peroxide value and TBARS. It was found that coatings themselves reduced oxidation and bacterial growth, and that incorporation of EO further improve these effects. From the results, it was evident that lipid peroxidation was influenced by the coating, probably because it reduced the permeability to O₂. The activity of the horsemint EO, which has no antioxidant components, may be due to an increase of the barrier effect (Heydari *et al.*, 2015).

9.6 Conclusions

From the above examples we showed that certain EOs and EOs components have the potential for being applied as antioxidants in real food. Amongst EOs, the most active are those of thymus, oregano and clove. Regarding the EOs components, the best antioxidant activity is shown by phenols (thymol, carvacrol, and eugenol) and by some non-phenolic compounds, such as γ -terpinene. On the other hand, there is the need for further investigations aimed at finding the best practises for their use and at clarifying their possible drawbacks in terms of toxicity and allergic induction.

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10

Principles of Sensory Evaluation in Foods Containing Essential Oil

Emma Mani-López, Ana Cecilia Lorenzo-Leal, Enrique Palou and Aurelio López-Malo

Departamento de Ingeniería Química y Alimentos, Universidad de las Américas Puebla, Puebla, Mexico

10.1 Introduction

One of the most important goals of the food industry is to understand how food affects consumer's senses and acceptance. That is why consumer reaction, perceived by the five senses, is considered a vital measure in food development. Since senses can't be substituted with any apparatus for food analysis, humans are utilised as test subjects during several product development steps, especially for evaluating product quality and acceptability (Edelstein, 2014).

The term 'organoleptic testing' was first used to denote an objective measurement of sensory attributes in the late 1990s, but in reality those tests were subjective, had few judges (tasters) and several interpretations were open to bias. In the early 1900s, ritualistic schemes for grading tea, wine, coffee, fish, butter and meat gave rise to professional tasters and consultants to beverage, food and cosmetic industries. Through the years, scientists have developed, formalised, structured and codified methodology for sensory analysis, continuing the development of new methods and refining existing ones (Meilgaard *et al.*, 2016).

Sensory analysis is known to apply the scientific method to identify, measure, evoke, analyse and interpret perceived attributes of a product. Like other assessable methods, sensory analysis is permanently implicated with precision, accuracy and sensitivity, as well as preventing false-positive results (Lawless & Heymann, 2010; Stone *et al.*, 2012; Edelstein, 2014). For a reliable sensory test, the sensory analyst has to fine-tune the following four factors: define the problem or item to be measured, design the appropriate test by minimising the number of tests required to produce the expected result, train the panelists (judges) and interpret the results by applying appropriate statistics (Edelstein, 2014).

Generally, panelists in sensory evaluations are usually placed in individual booths, tables or cubicles, so that the judgements given are theirs and do not imitate the choices of others. Samples are identified with random numbers and in a uniform fashion, with

the objective of minimising judgement based upon identifiers, but rather on judges' sensory experience. Also the environment used to evaluate the food or beverages has to be carefully controlled to avoid errors (Lawless & Heymann, 2010; Edelstein, 2014).

10.2 Sensory Aspects of Essential Oils

Essential oils are extracted from different parts of plants (flowers, seeds, leaves, fruits, roots and rhizomes, amongst others), and they could have antiviral, antibacterial, antifungal and/or insecticide properties, amongst others (Burt, 2004; Seung-Joo *et al.*, 2005). Essential oils usually differ in their odours, refractive indices, optical and specific rotation properties. They are soluble in ether, alcohol and almost all organic solvents, but immiscible with water. Some of their main components include hydrocarbons, alcohols, ketones, aldehydes, ethers, esters and oxides. An essential oil may contain approximately 200 components related to its odour and flavour, and the absence of only one component could mean a change in the aroma and/or its properties (Noudogbessi *et al.*, 2012).

Essential oils and their components are used as food additives, and their principal function is to impart desirable flavours and aromas to different foods, beverages and confectionary products. Generally, the most important aspects to sensory analyse essential oils are visual quality, colour, odour and taste (Mariod, 2016). For example, sweet basil (*Ocimum basilicum* L.) is currently used to develop unique flavours in salads and other foods (pizzas, meats and soups, amongst others) due to its phenolic compounds (linalool as well as rosmarinic and caffeic acids); which have also important antioxidant capacities (Seung-Joo *et al.*, 2005; Calín-Sánchez *et al.*, 2012).

Some plants, their extracts, essential oils, and/or active components have antimicrobial and antioxidant effects. Essential oils proved to have antimicrobial or antioxidant properties against different microorganisms in culture media and model-food systems. However, when essential oils are tested in foods, generally, it requires 1 to 3% more essential oil to achieve the same effect, which may affect foods' sensory acceptabilities (Seung-Joo *et al.*, 2005; Rivera-Calo *et al.*, 2015). Usually essential oils are more expensive than synthetic additives, could present an interaction with some food ingredients, and their strong odour could be unacceptable to consumers. Therefore, practical applications of essential oils in foods may be limited (Aloui *et al.*, 2014; Kahraman *et al.*, 2015; Moon & Rhee, 2016). Some concentrations of essential oils, such as those obtained from oranges, could affect the flavour and aroma of most foods at very low concentrations (Espina *et al.*, 2014); being essential oils from citrus fruits, important sources for fragrances and scents, having approximately 25% of the global aroma market, applied widely in food, cosmetic, and pharmaceutical industries (Sun *et al.*, 2014).

Since generally higher concentration of essential oils are required when they are added to foods, some options that could be performed to overcome changes on food sensory attributes are essential oil could be used as antimicrobial and flavour component at the same time; they may be used in products that already have strong flavour so the presence of the essential oil is masked; use the most active components rather than the whole oil (taking into account that the antimicrobial or antioxidant activity of the oil could be due to the interaction amongst different components); develop a synergic combination with various essential oils reducing the concentration to be used of each

essential oil; consider potential applications that don't require direct incorporation of the essential oils into foods (like encapsulation or edible films); or they can be utilised and combined with other processes such as low pH or refrigeration temperatures (Barry-Ryan & Bourke, 2012).

10.2.1 Selected Examples

Black pepper is a popular spice around the world that is used as flavouring, as well as to preserve processed foods. The value of this spice as food additive depends on its essential oil. More than 80 constituents have been identified in pepper essential oils; there could be differences in the content of oil, as well as in colour, flavour, aroma and chemical composition due to different varieties of the spice (Mamatha *et al.*, 2008). Mamatha *et al.* (2008) described the flavour and odour of peppers from different cultivars by means of sensory evaluation, gas chromatography-mass spectrometry (GC-MS), and electronic nose analyses. Flavour profile (evaluated aspects are depicted in Table 10.1) of studied essential oils showed that three of the analysed samples (panniyur, panniur-5, and a commercial one), had a high intensity of *pepper-like* attribute No. 7, while attributes *green mango-like* (No. 11) and *turmeric powder* (No. 20) characterised the Balankotta sample. Furthermore, the odour profile supported the flavour profile data while the GC-MS and electronic nose analyses complemented the obtained sensory flavour profile.

On the other hand, Calín-Sánchez *et al.* (2012) performed a sensory analysis with a trained panel of eight judges (competent in descriptive sensory assessment of fruits, herbs and vegetables) to distinguish the intensities of the main aroma elements of fresh, convective (CO, 40 °C) and vacuum (VC, 240 W) microwaved dried samples of sweet basil. The panelist evaluated sweet basil samples (from different regions of Spain) that were fresh and dry and concluded that 10 main attributes (fresh sweet basil, vegetable,

Table 10.1 Aspects evaluated for sensory flavour profile of pepper essential oils (Source: Adapted from Mamatha *et al.*, 2008).

No. attribute	Attribute	No. attribute	Attribute
1	Refreshing	13	Menthol, myrcene
2	Herbaceous	14	Isopropyl-quimoline
3	Spicy	15	Piperine
4	Earthy	16	Isopropyl-quinoline
5	Lemony	17	Citral
6	Aromatic	18	Benzaldehyde
7	Pepper-like	19	Pepper powder
8	Turmeric	20	Turmeric powder
9	Camphory	21	DL-camphor
10	Citrusy	22	Limonene
11	Green mango-like	23	Iso-cyclocitral
12	Fruity	24	Aldehyde

herbaceous, balsamic, spice, hay-like, sweet, earthy, woody and infusion) could be used to describe the odour. Results of the descriptive sensory analysis showed a significant difference ($p < 0.01$) on the increases of the following attributes on the CO and VM samples: spicy, hay-like, sweet, earthy, woody and infusion. The decreasing attributes resulting from drying were fresh, floral and herbaceous.

Similar to the previous study, Bonfantia *et al.* (2012) utilise oregano essential oils (five different samples, four cultivated in different areas of Sicily as well as a commercial one) in order to characterise and sensory evaluate them. The essential oil of the different samples was obtained by hydrodistillation and they were chemically characterised by gas chromatography-flame ionisation-mass spectra. The Sicilian samples had a comparable chemical composition, being the main components thymol and γ -terpinene, followed by p -cimene, α -terpinene, carvacrol and thymol methyl ethers; while in the commercial sample carvacrol was the main component, followed by p -cimene, γ -terpinene and thymol. Regarding the sensory profile, the attributes evaluated were colour intensity, freshness, hay odour, off-odour, bitter, oregano typical flavour, hay flavour, mint flavour, off-flavour and colour intensity. Sicilian oregano samples were very homogenous (as expected by their similar chemical profiles), showing only that only one sample had a higher mean score of colour intensity; although the commercial oregano oil had a greater intensity in 'hay odour and flavour', bitterness, off-flavour and off-odour, as well as lower scores for the following attributes: freshness, colour intensity, typical oregano flavour and mint flavour.

10.3 Desirable Applications of Essential Oils and Their Relation with Sensory Analysis

10.3.1 Antimicrobial Applications

There has been an increasing interest on natural additives that can be applied to food, because of the demand of consumers for fresh, natural, free of chemical-additives and wholesome food products. Some of the natural antimicrobial compounds available as food antimicrobial alternatives include several herbs and species, as well as their extracts and essential oils (Skandamis & Nychas, 2001; Valero & Francés, 2006; Gonçalves-Cattelan *et al.*, 2015; Moon & Rhee, 2016; Nielsen & Rios, 2000). It is worth to mention that essential oils are classified as *generally recognised as safe* (GRAS) by the FDA (U.S. FDA, 2016).

10.3.2 Antioxidant Applications

The antioxidant activity of essential oils also has been intensely investigated. Generally, research has aimed on studying crude extracts' antioxidant activities; using different solvents (acetone, methanol, or water). On the study made by Seung-Joo *et al.* (2005), scent components in thyme (*Thymus vulgaris*) and basil (*Ocimum basilicum*) leaves' extracts were analysed by GC and GM-MS. Being the major aroma components of basil extracts linalool, propenyl, benzene, methyl cinnamate, eugenol and 1,8-cineole, and the major volatile components in *Thymus vulgaris* were thymol, carvacrol, linalool, α -terpineol and 1,8-cineole. Further, eugenol, thymol and carvacrol exhibited were better antioxidant agents than the other identified components.

Ramadan *et al.* (2013) evaluated the antioxidant activity of thyme, basil and marjoram essential oils with the purpose of using them as natural flavouring agents (with antioxidant activity) in ice cream. Marjoram essential oil presented a greater antioxidant activity (established by the phosphomolybdenum method) than basil and thyme essential oils. A sensory analysis was implemented using test scores for body and texture, flavour, resistance to melting, appearance and overall acceptability, by means of a scale from 1 (representing very poor) to 9 (being excellent). High scores were assigned to quality attributes. The addition of essential oils did not have a significant ($p > 0.05$) effect on texture, body, resistance to melting and appearance scores. But, flavour scores were higher ($p \leq 0.05$) for ice creams with marjoram or thyme essential oils. Also the ice cream flavoured with marjoram essential oil was superior in its sensory acceptability than the other tested ice creams.

10.3.3 Other Applications

Textile fabrics with antimicrobial and aromatic properties could be manufactured by the application of chitosan film coated with rosemary essential oil, at the surface level of fabrics. Sensory evaluation in this study was carried out with samples treated with rosemary essential oil, which were stored at room temperature and smelled every five days. As expected, the essential oil had antibacterial activity and the scent intensity of the treated samples increased with treatment concentration. The smell persisted for 15 days and then gradually decreased (Muresan *et al.*, 2009).

On the other hand, carpet cleaners prepared with wood oils were evaluated by perfumers. Seven male perfumers were asked to spontaneously describe their impression regarding the scents of different essential oil concentrations, carried out the sensory analysis. Results showed that the carpet cleaner without oil (control) was described and rated with slight smell or no smell, while the cleaners with different essential oil concentrations were between moderate and strong smell. Also, the freely description included the following aspects: woody, citrus, medical, pine-resin, resin, and earthy; being woody the most mentioned attribute (Yamamoto *et al.*, 1998).

10.4 The Relationship Between Composition of Essential Oils and Sensory Properties

Composition of essential oils depends on what part of the plant is used, conditions of plant growth, drying methods and extraction processes, amongst other factors (Rivera-Calo *et al.*, 2015). Some examples of such factors are presented below.

Mamatha *et al.* (2008) chemically characterised essential oils of black pepper from different cultivars (Balankotta, panniyur, panniyur-5, and a commercial sample) by GC-MS, demonstrating that the sample of Balankotta was different from the panniyur, panniyur-5 and commercial samples; this can be explained by its high content of ρ -cymene, component which the other tested samples did not had. This difference was also reflected in the flavour profiles, showing different distinguishing attributes amongst tested samples.

Citrus peels volatile extracts have shown to be mainly composed of sesquiterpenes and monoterpenes; limonene appears to be the most abundant amongst other

components, such as aldehydes, ketones, acids, alcohols and esters. Furthermore, the quality of the volatile extracts could vary by the citrus species and preparing method. For example, red blush grapefruit (*Citrus fruit*) has been identified with 67 volatile chemicals while pomelo (*Citrus maxima*) with 52 volatiles. It is known that the preparation or extraction methods, affect the quality and favor profiles of citrus oils (Sun *et al.*, 2014).

Sun *et al.* (2014) analysed different odour notes of diluted volatile extracts from pomelo (*Citrus maxima*) peel, obtained by cold pressing (CP), simultaneous distillation and extraction (SDE), microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE) and water distillation (WD). A nine-point intensity scale was used to evaluate the following aspects: sweet, lemon, pomelo flavour, green/herbaceous odour, fermented or overripe and alcoholic or pungent odour; in which 0 meant unperceived attribute intensity and 9 very strong attribute intensity. Results from CP samples showed that herbaceous and pomelo-like notes were dominant, green was strong and fermented/overripe was the weakest attribute. Unlike CP samples, WD and SDE samples presented very similar sensory profiles with strong fermented/overripe and alcohol/pungent attributes. Finally, MAE and UAE samples also were similar to each other, with green and pomelo-like odour notes; while SFE samples had sweet and musk notes as dominant. Furthermore, these samples were analysed by gas GC-MS to determine their chemical composition. The volatile extracts of MAE, CP, SDE, UAE, WD and SFE had 47, 49, 44, 45, 49 and 40 volatile compounds, respectively; showing that limonene and β -myrcene (Figure 10.1) were the main components of tested citrus essential oils, proving that the extraction method could also be a factor that affects both, chemical composition and sensory profile of the essential oils.

A similar study was published by Bousbia *et al.* (2009) where samples of rosemary essential oils, extracted by hydrodistillation (HD) or by microwave hydrodiffusion and gravity (MHG) were analysed. A sensory analysis was performed with 12 panelists, whom concluded that the sample extracted by MHG had a pale yellow colour, a fresh, light, freshly, camphorated with slight note of citrus and close to fresh aromatic herb odour and a liquid movable aspect. For the sample extracted by HD, the colour was also pale yellow, while its odour was freshly camphorated and citrus, with a boiled odour and different from fresh fruit and also had a liquid movable aspect; consequently, ascertaining that the extraction method affects the essential oils' quality.

Also, when different parts of the same plants are used to extract essential oil, the composition and sensory profile could be affected, even though the origin of the essential oil is from the same plant. Such a thing happened in essential oils obtained from different

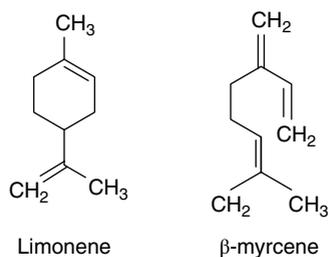


Figure 10.1 Main components of citrus essential oils.

basil parts (leaves, flowers, stems, whole plant or leaves-flowers). The essential oils were classified into five clusters by a ranking test (preference) of aromas, and by an odour assessment of volatile compounds. The chemical composition was performed by GC and sensory evaluation, which was performed to determine the distinctive scent of each volatile component of the diverse basil essential oils by trained panelists. Some of the major components and their odour description are presented in Table 10.2. Amongst the samples, the odour of leaves-flowers essential oil was the most desirable, while the stem sample was the least preferred. Also, the major components present in the essential oils (but in different percentage) were methyl-chavicol, followed by 1,8-cineole, and cis- β -ocimene (Lee-Yan *et al.*, 1991).

Bernhardt *et al.* (2015) made a correlation between sensory attributes and the chemical compounds for 12 different samples of basil (*Ocimum basilicum*) essential oils. All samples had different percentages of chemical constituents, being the principal ones in almost all the tested samples linalool, methyl-chavicol, neral, geranial and 1,8 cineole. The intense lemon odour was strongly correlated to the presence of neral, geranial, isocitral, octanal, turpentine and β -cubebene. The intense sweet odour was correlated to the presence of furanoid, nerol, neryl-acetate and caryophyllene oxide. The spicy odour was related to eugenol, sabinene, β -caryophyllene, α -guiene and cis- α -bisabolene; while the hay-like odour was due to β -pinene. These authors concluded that detection of essential oil compounds could vary a lot, because sensory panelists can detect components at low concentrations more intense than some constituents at high concentrations. To avoid this kind of error, it is suggested to use both, instrumental and sensory, assessment methods.

In the case of *Chrysanthemum* essential oils, six sensory attributes for five different essential oils, were evaluated: floral, grassy, woody, sour, fruity and minty. A total of 38 volatile compounds were detected in this essential oil by GC-MS and a relationship between sensory evaluation and odour-active components was achieved. The floral attribute was related to the presence of linalool, while the grassy attribute was related with α -pinene, camphene, cis-ocimene, α -fenchone and thymol. The woody note, was related to sabinene, germacrene and cinnamaldehyde; along with the minty note to 4-terpineol and carvone. The fruity and sour attributes were weak notes also detected in the sensory test. This study, as the one reported by Lee-Yan *et al.* (1991), concluded that different composition of the essential oils could impact the sensory analysis (Xiao *et al.*, 2016a). Another study published by Miyazawa *et al.* (2011), reports about the aroma analysis of diluted gamazumi (*Viburnum dilatatum*) essential oil and the FD factor of

Table 10.2 Some of the major components of selected basil essential oils and their odour description (Source: Adapted from Lee-Yan *et al.*, 1991).

Compound	Odour description
Methyl-chavicol	Fragrant, sweet, cooling, fresh, minty
1,8 cineole	Fragrant, sweet, cooling, fresh, slightly green, minty.
cis- β -ocimene	Fragrant, sweet, cooling, fresh, slightly green, minty.
Methyl-eugenol	Fragrant, sweet, fresh, slightly green.
Trans- β -farnesene	Cooling, fresh, minty, slightly green.

its components. The FD factor was calculated, as the proportion of the concentration of the note in the initial essential oil to its concentration in the diluted sample in which the odour is still detectable by GC. The highest FD factors were from nonanal, linalool and 3Z-hecanel, therefore, these compounds were considered to be the main aromatic compounds of the leaf oil. There were 24 different components found in the essential oil by GC. It is also important to mention that with this correlation it was also found that C6 compounds and 2-pentyl furan produced the green odour while linalool, β -ionene and eugenol produced the sweet odour.

Extraction of essential oils from lavender flowers was achieved by eight different extraction techniques: hydrodistillation (HD), steam distillation (SD), turbo-hydrodistillation (THD), ultrasound-assisted extraction (US-SD), microwave-hydrodistillation (ISMH), microwave steam distillation (MSD) microwave hydrodiffusion and gravity (MHG) and microwave steam diffusion; the composition and sensory analysis of these essential oils, were performed to compare them. These essential oils were evaluated by four aspects: musk scent, flower odour, herbaceous scent and camphor smell. Musk scent was detected in the THD extracted oil while the other tested oils did not have significant ($p > 0.05$) differences between their herbaceous scent, flower odour and camphor smell. It is worth to mention that the studied microwave techniques generated the same sensory perception of their oils (Périno-Issartier *et al.*, 2013).

10.5 Factors Influencing Sensory Measurements

For a suitable sensory analysis, it is necessary to consider panelists as measurement instruments, slightly variable over time, amongst themselves and susceptible to bias (Meilgaard *et al.*, 2016). This raises the importance of understanding the factors influencing their sensory verdicts; some of these factors are presented below.

10.5.1 Physiological Factors

Amongst main physiological factors are adaptation and enhancement (suppression). Adaptation involves sensitivity adjustment to a given stimulus as a result of sustained experience to a particular stimulus. In sensory analysis, this effect is an undesirable cause for variability and intensity of ratings (Meilgaard *et al.*, 2016). On the other hand, enhancement includes the interaction of stimulations presented concurrently as mixes. The increased of the perception on the intensity of a substance, caused by the presence of another substance, is called the effect of enhancement. Synergy is the effect of intensifying the combined intensity of studied substances by the presence of one substance, such that the perceived mixture intensity is greater than the sum of the individual components. Finally, suppression is the effect that represents one substance presence diminishing the noticed intensity of two, three or more substances mixture (Meilgaard *et al.*, 2016).

10.5.2 Psychological Considerations

The psychological errors that could happen during sensory analysis of essential oils are presented in Table 10.3. This error should be always avoided so validity of the tests won't be affected.

Table 10.3 Psychological errors influencing sensory measurements.

Psychological errors	Description	Control of the error/factor
Expectation error	This error could happen when information about the samples is given to the panelists prior to the test. This information can influence results, because panelists generate preconceived ideas and they find what they are expected to find. Expectation error can destroy the validity of a test and must be avoided.	<ul style="list-style-type: none"> ● Giving enough information to the panelists, for them to perform the task. ● Coding the samples so the panelists won't be able to identify them. ● Persons that are involved with the test, shall not be included on the panel. ● Keeping the testing area free of distractions and noises. ● Separating the test area from the food preparation section.
Suggestion effect	There could be an important influence in the response of a panelist caused by the reaction of other panel members.	<ul style="list-style-type: none"> ● Separating panelists by using individual booths, tables, or cubicles. ● Not allowing discussion during testing.
Stimulus error	This happens when panelists are influenced by irrelevant sample differences, such as volume, size, and colour; or style and colour of the container.	<ul style="list-style-type: none"> ● It is important that the samples are always prepared, sampled, and served under identical conditions. ● The differences in colour could be hidden by filters or coloured lights. ● Avoid leaving irrelevant (as well as relevant) cues.
Positional bias or order effect	The panelist judgement could be influenced by the order in which the samples are evaluated. The first sample of a series of samples, is usually highly scored than expected, regardless of the product.	<ul style="list-style-type: none"> ● Samples are recommended to be served in randomized order to ensure that they are presented in different positions.
Central tendency error	This error is shown when panelists score products in the midrange of a scale, usually panelists avoid the extreme ends of the scale.	<ul style="list-style-type: none"> ● This effect is minimized when samples are presented in randomized order. ● Familiarize panelists with the test methods, products, and testing procedures.
Contrast and convergence errors	When two samples are markedly different from each other, the error of contrast occurs, and panelists may exaggerate the difference in their scores. The opposite effect is convergence, and is brought about by masking smaller differences between samples.	<ul style="list-style-type: none"> ● Randomize the order of the presentation of samples for each panelist.
Logical error	Logical error occurs when two or more characteristics of a sample are related in the minds of the panelists.	<ul style="list-style-type: none"> ● Keeping the samples uniform. ● Masking the possible differences amongst the samples.

(Continued)

Table 10.3 (Continued)

Psychological errors	Description	Control of the error/factor
Error of habituation	A tendency to continue to give the same response, when a series of slowly increasing or decreasing stimuli are presented.	<ul style="list-style-type: none"> ● Vary the type of product or masking the samples.
Halo effect	<p>When more than one characteristic of a sample is evaluated, ratings could influence one another.</p> <p>When simultaneous scoring of various flavour aspects and overall acceptability could generate different results than if each attribute is evaluated separately.</p>	<ul style="list-style-type: none"> ● Present separate sets of samples for evaluation of attributes.
Lack of motivation	It is related to the degree of effort a panelist will make to discern a subtle difference, to find the proper term for a given impression, or to be consistent. It is worth mentioning that an interested panelist is always more efficient.	<ul style="list-style-type: none"> ● It could be minimized by having a controlled test.

Warner & Michael, 1995; Kemp *et al.*, 2009; Lawless & Heymann, 2010; Kilcast & Subramaniam, 2011; Meilgaard *et al.*, 2016.

10.5.3 Other Factors

In sensory analysis, it is also important to control the climate. The discussion areas as well as where the panelist are, should be climate controlled and odour-free, having excellent ventilation. Also, it is important to check that cleaning supplies used in the area do not add odours. Sensory analysis requires silence, so it is necessary to avoid any kind of noise, even the ones generated from mechanical systems like freezers, air conditioners and/or processing equipment. Temperature and relative humidity of the area should be between 20 and 22 °C and 50 to 55%, respectively, providing a comfortable environment for the panelist and preventing distractions. Illumination should be between 300 and 500 lux at the sensory tables' superficies. In some cases, it could be controlled with a dimmer switch up to 700–800 lux (Lawless & Heymann, 2010).

Another factor that could affect sensory evaluation is poor physical condition of the panelist. Panelists should be excused from the test when they are suffering of fever or common cold, have poor dental hygiene or gingivitis and when they have heavy pressures from work or emotional upsets (Meilgaard *et al.*, 2016).

Other important considerations that must be transmitted to the panelists are when panelists are smokers, they should stop smoking 30 to 60 minutes prior to the test; also strong coffee drinking could paralyse the taste for up to one hour; and the test should not be performed the first two hours after main meals (Meilgaard *et al.*, 2016).

10.6 Selection and Training of Panelists

Trained panels are expensive for companies that train, maintain and utilise them. For companies or universities that rely on personnel: (1) a big enough pool of candidates is necessary for selection of panelists, (2) sensory staff is necessary to apply selection, training and uphold procedures; including a panel leader and a technician, and (3) a skilled person to lead the training is a must. With regards to facilities, a space for selection, training and panel's testing area, as well as sensory testing facilities that have proper environmental controls, near to product preparation are required. Personnel, hardware and software required to gather and examine the panel's data are also essential. Two technical guides are available for selection and training of panels, the *Guidelines for the Selection and Training of Sensory Panel Members* from ASTM Special Technical Publication 758 (1981) and the *Guide for Selection and Training of Assessors* from ISO (1993). Most published research is based full on these guides.

10.6.1 Panelists for Descriptive Testing

Descriptive analysis panels are groups of well-trained persons with sharpened senses, as well as consistent, reliable and homogeneous terminology and scales (Stoer *et al.*, 2002) that should be learned during training; thresholds of detection and intensity of sensory attributes are also defined. It is expected that trained panelists are able to detect and describe all the perceived aromas, appearances, flavours, textures and so on, thus they should be sensitive persons or more sensible than an average person. Selection of panelists begins with a screening from a group of candidates, and specific abilities are delimited according with type of required descriptive test. According with Stoer *et al.* (2002) key standards used for establishing the finest panelists for evaluation of foods comprise:

- a) Ability for understanding abstract conceptions and put on them for the appraisal of sensory attributes since they are asked to grasp concepts in terms of both, vocabulary and scoring.
- b) Healthiness of panelists is essential. Persons with allergies or sensible to particular ingredients or with diseases such as diabetes, hypertension, sinusitis, central nervous disorders or reduced nerve sensitivity should be rejected.
- c) Availability of schedule in order to meet 50–100 hours of training.
- d) High interest in foods and sensory work, due to the training demands that require great attention and willpower, as well as numerous tastings.
- e) Sensory keenness, since individuals involved in sensory training have to be able to distinguish changes, alterations, or modifications, as well as selected attributes in food products.
- f) Verbal/descriptive talents are necessary since panelists' task is to clearly explain the noticed attributes.
- g) Group dynamic abilities are important in order to interchange ideas to resolve opinions differences, scores, or attributes in every panel meeting as well as in day-to-day descriptive testing.

Depending on the required test, different methods are used as screening means to determine the initial qualifications of candidates, the application of a survey to establish

aptitude, health, availability, verbal communication skills and some descriptive capabilities could be the beginning of panel selection (Meilgaard *et al.*, 2007). Selection of candidates based on their answers carries out them to the next steps, insight screening and dialogues. In a session (one hour), candidates are requested to perform a basic taste identification exercise, selected multiple triangle test examples or practise tests, and a taste ranking (basic) exercise (Stoer *et al.*, 2002). Some criteria to choose candidates include: for detection acuity test select the subjects who achieve 50–60% (in triangle tests) acceptable responses or in duo-trio tests 70–80%; for description acuity test include subjects that are capable to describe at least 80% of the stimuli by means of chemical, common, or related terms (*i.e.*, orange, floral, ginger, peppermint, anise, vanilla, cinnamon, and clove, amongst others). In ranking screening, aspirants must rank or rate several samples on a key attribute, aspirants were selected from those who rate all samples in correct order for at least 80% of the studied attributes. In the personal interview, the panel leader should confirm candidate's interest in sensory analysis; communication skills and general personality should be also considered. Candidates with little interest, hostile or timid personalities might be excluded. A mock panel also is useful to select the best candidates of the group (Meilgaard *et al.*, 2007).

10.6.2 Trained Descriptive Panel

Time required to train a descriptive panel is between 40 and 120 hours and depends on the complexity of the product to be tested, number of attributes to assess and requirements for validity and reproducibility. The first stage includes terminology development from chemical/physical principles that rule the perception of every attribute in the selected product. At the end of this phase, panel has developed techniques for assessment and lexicon (definitions and references) for the specific product. The second phase is an introduction to descriptive scales by means of a collection of samples that represents three or five different intensities of every attribute, the panel leader emphasises both sensory characteristics and the scales (attributes and intensities) in order for the panel to begin seeing the descriptive process (terms and numbers) to define whichever product in the studied group. The third stage is initial practise (detailed vocabulary development); a full collection of products, prototypes or example products are presented to the panel as a reference that is to be described with several descriptors and finally panel and leader choose products and prototypes, as well as outside references that represent suitable examples of the selected terms. The fourth step is related to small product differences, panel leader presents products with smaller differences (process or ingredient variation) in order for panelists to refine procedures for assessment and lexicon with definitions and references that enable identifying and describing product dissimilarities. The last step is a final practise, and the panel continues to test and depict several products during the final rehearsal now with real-world testing products. In addition, performance of the panel has to be evaluated based with regards to bias or variability.

Panelist maintenance, feedback, rewards and motivation are maintained during the panel life in order to ensure adequate performance of the trained panel (Meilgaard *et al.*, 2007).

Training a panel for EO descriptive tests should follow the standardised training methods described above. When a specific food with an EO is evaluated also standardised methods are followed or adapted. For olive oil recognised important characteristics,

a sensory analyst trained preselected individuals for 15 sessions of two hours until intensities and descriptors were in accordance with lexicon for extra virgin olive oil (EVOO) while final testing was directed by the panel leader. Descriptors used in this training are shown in Table 10.4. Intensity scores were created during training; panelists' performance was checked by clearly defined reference samples, variance scores were revised in order to verify panelists' skills and consistency. A calibrated panel was achieved when panelists' variance of EVOO attributes was $\leq 5\%$ (Asensio *et al.*, 2012). For ricotta cheese after a familiarisation session with sensory concepts, panelists acted together to create the lexicon to depict product attributes. Panelists also recognised references to be utilised to portray appearance, flavour and texture attributes. After that, intensity rating was estimated and utilised for each particular reference. In subsequent sessions, panelists reexamined descriptors, definitions, and standard references to sensory define ricotta cheese until panel calibration was achieved. A calibrated panel was obtained when the panel scores obtained a standard deviation within ten points' average. The trained panel was improved by means of quantitative descriptive analysis (QDA) and the SpectrumTM (Asensio *et al.*, 2014).

10.6.3 Selection and Training of Panelists for Discrimination Tests

General considerations regarding panel members' interest and health problems of candidates as mentioned for candidates for a descriptive test must be performed. Screening tests allow to select candidates with the ability to discriminate differences amongst samples or products and ability to distinguish intensities and differences in the evaluated characteristics. Three kinds of tests have to be taken into account in order to select the candidates that integrate the final panel (Meilgaard *et al.*, 2007):

- 1) Matching tests to establish a candidate's ability to discriminate differences;
- 2) Detection/discrimination tests to validate a candidate's capability to notice dissimilarities amongst similar products; and
- 3) Ranking/rating tests to establish candidate's aptitude to differentiate graded levels of intensity. For specifics, refer to Meilgaard *et al.* (2007).

10.7 Sample Preparation

Sensory analysts are frequently cautious to detail specific preparation methods to guarantee uniformity of samples for assessment, since sample preparation should be done in uniform way (form and sample quantity, brand and material of containers, dimensions, *etc.*) in order to minimise the environmental aspects' effect on panelist perception. Also temperature has to be considered when it affects sensorial perception, such as in the case of meats, milk and ice cream. Sometimes carriers are used to accompany samples; however, they should be avoided if the purpose of the sensory evaluation is to detect differences between samples or to identify specific taste attributes, such as foods added with EOs. Palate cleansing is also a critical factor since a correct elimination of residual materials from preceding samples ensures independent evaluation amongst samples. Instructions to panelists must be clear and succinct and preferably written. Labelling samples with three-digit codes of random numbers in order to avoid bias and sample order, according to the international best practises.

Table 10.4 Adjective descriptors used to describe sensory profile of selected essential oils and herbs.

Essential oil/herb	Adjective descriptors for trained	Scale	Purpose of the test	No. of panelists	References
Sage (<i>Salvia officinalis</i>) EO	Pine-like, fresh, resinous-piney, lime-like, citrus-like, lemon note, eucalyptus-like, sweet, weak floral, herbal, floral, sweet-piney, fresh-floral, minty, earthy, weak fruity, balsamic, woody, spicy, woody-spicy	—	Describe the aroma	3	Edris <i>et al.</i> , 2007
Pepper (<i>Piper nigrum</i> L.) EO	Refreshing, herbaceous, spicy, earthy, lemony, aromatic, pepper-like, turmeric, camphory, citrusy, green mango-like, fruity	Structured scale of 15 cm for QDA technique	Asses the odour and flavour quality of three cultivars	10–12	Mamatha <i>et al.</i> , 2008
Oregano (<i>Origanum vulgare</i>) EO	Fresh oregano, vegetable, pine, herbaceous, balsamic, spice, hay-like, sweet, earthy, woody, infusion	11 points scale (0 = non perceptible intensity, 10 = extremely high intensity)	Discriminate the intensities of the main aromatic characteristic of oregano dried by three methods	10	Figiel <i>et al.</i> , 2010
Rosemary (<i>Rosmarinus officinalis</i>) EO	Fresh rosemary, vegetable, pine, herbaceous, balsamic, spice, hay-like, sweet, earthy, woody, infusion	11 points scale (0 = non perceptible intensity, 10 = extremely high intensity)	Discriminate the intensities of the main aromatic characteristic of rosemary dried by three methods	10	Szumny <i>et al.</i> , 2010
Cumin EO	Citrusy, cumin-like, floral, green, herbal, cooling, earthy, spicy, sweet	Structured scale of 15 cm for QDA technique	Describe the aroma compounds of cumin from different regions	—	Ravi <i>et al.</i> , 2013
Thyme (<i>Thymus vulgaris</i> L.) EO	Fresh thyme, vegetable, herbaceous, balsamic, spice, hay-like, sweet, earthy, woody, infusion	11 points scale (0 = non perceptible intensity, 10 = extremely high intensity)	Discriminate the intensities of the main aromatic characteristic of thyme dried by three methods	8	Calín-Sánchez <i>et al.</i> , 2013

<i>Citrus maxima</i> peel EO	Sweet, lemon, pummel flavour, green/ herbaceous odours, fermented/override, alcohol/pungent	9 points scale (0 unperceived attribute, 9 very strong attribute)	Investigate the aroma and volatiles of the volatile extract of pummel peel using different extraction methods	3 for the first stage and 15 for the second	Sun <i>et al.</i> , 2014
Sweet orange EO	Green, fruity, peely, fatty, floral, woody	10 point interval scale (0 = none, 9 = extremely)	Describe the aroma attributes and classify the aroma notes of different sweet orange EOs	8	Xiao <i>et al.</i> , 2016b
<i>Chrysanthemum</i> EO	Floral, woody, grassy, fruity, sour, minty	10 points (0-none, 9-extra strong)	Characterize 5 EO from different manufactures	15	Xiao <i>et al.</i> , 2016a
Lavender (<i>Lavandula</i> <i>hybrida</i> L.) EO	Floral lavender, herbaceous, camphoraceous, woody, spicy pepper, fruity, fatty/aldehyde, sweet/candy, soapy, off-note	Scale of 0–3, 0: not present, 1: slight intensity, 2: average intensity, 3: high intensity	Discriminate the intensities of the main aromatic characteristic of lavender EO obtained by 10 extraction techniques	1	Filly <i>et al.</i> , 2016
<i>Ocimum</i> <i>basilicum</i> L.	Fresh sweet basil, vegetable, herbaceous, balsamic, spice, hay-like, sweet, earthy, woody, infusion	0–10 (0 = non perceptible intensity and 10 = extremely high intensity)	Quality and volatile composition of sweet basil dried by three different methods	8	Calín- Sánchez <i>et al.</i> , 2012
<i>Ocimum</i> <i>basilicum</i> L.	Global odour, anise odour, lemon odour, hay-like odour, spicy odour, sweet odour, basil odour, pine rein odour	0–100 points	Compared qualitative parameters of aroma profiles of 12 cultivars	10	Bernhardt <i>et al.</i> , 2015
<i>Origanum</i> <i>majorana</i> L.	Fresh marjoram, vegetable, pine, herbaceous, balsamic, citric, spice, hay-like, sweet, earthy, woody, infusion	0 to 10, where 0-none or not perceptible intensity, and 10-extremely high intensity	Quantify the intensity of the main aroma attributes of fresh and dried marjoram	7	Calín- Sánchez <i>et al.</i> , 2015

EO = essential oil, QDA = quantitative descriptive analysis.

Due to volatile nature of the EOs, preparation of the sample must be specific and careful; some examples of sample preparation for sensory evaluation of EOs are presented next. For odour analysis of pepper EO, 0.1 g was taken into an Erlenmeyer (250 ml) and covered with a cotton layer, and then Erlenmeyer was stoppered for 15 min to allow the volatile accumulation in the headspace (Mamatha *et al.*, 2008). Panelists had to smell the headspace and differentiate odour notes. For cumin EO, a volume of 20 μ L in a 50-ml glass bottle plugged with cotton wool was enough to adequately carry out the odour test (Ravi *et al.*, 2013). Other authors diluted the volatile extract (EO) before evaluation took place. Pummelo peel (*Citrus maxima*) EO (100 μ L) was diluted with alcohol (400 μ L) and mixed, then 10 μ L of the diluted sample was added onto a stripe for test fragrances, prior to the sensory evaluation the stripe was dried for two min (Sun *et al.*, 2014); this sample preparation was used to evaluate the aroma and volatiles of pummelo peel EO extracted by six different methods. The odour profile was differentiated into three types of notes: a top note released within 3 min, a middle note released up to 1 h and a base note that was released after 1 h. In addition, some authors simply put one drop of EO on a commercial odour-strip and panelists smell the odour. Furthermore, a smelling strip was wet with the EO about 1 cm (in one end) three times, panelist quickly sniffed the sensing the strip and then odour source was removed; before other sample is smelled clean air for at least 20 s had to be breathed (Xiao *et al.*, 2016b). Another simple way to present EO is to put a specific volume in beakers (100 ml) with lids and set upright them at room temperature for 30 min prior to analyses (Figiel *et al.*, 2010); also 10% of ethanol can be used to dilute sample of EO prior to sensory analysis (Filly *et al.*, 2016). Another way was to place 0.5 ml of EO in slides of absorbent paper (10 x 0.5 cm) coded with three digit random numbers (Asensio *et al.*, 2015) and give to panelists in order to identify attributes of the tested odour profile.

Samples of pepper EO for sensory flavour assessment were prepared using starch gruel as medium. Preparation of gruel consisted in dispersing of cornstarch (2 g) in 100 ml of water with 0.25 g of table salt; the dispersion was heated until obtaining a uniform gelatinised liquid and then 0.02 g of EO was added and mixed to obtain homogeneous dispersion. The selection of a proper medium for sensory assessment of EOs is a critical step when a specific note is accentuated since if an inadequate medium is used and the note is not perceived, a false absence could be reported (Mamatha *et al.*, 2008).

Sensory evaluation of EOs can take place incorporated in foods; therefore, researchers or sensory analysts will want to know the impact of the EOs in an specific food or the organoleptic changes in foods when EOs are added. Currently, EOs in foods act as antimicrobials, antioxidants, and/or as flavourings; thus, specific amounts are required in order to achieve the preservative action or enhanced flavour. Therefore, sensory analysis of the food should be carried out. Sample preparation is going to be different from the one performed for sensory analysis of EOs; however, size, form, and containers have to be uniform. Some studies showed that different containers or placement of food in the container had an impact in the consumer perception of the product (Michel *et al.*, 2015; Wan *et al.*, 2015). Cooking method also influence in the attributes' perception and their intensity (Bach *et al.*, 2013); thus, unsystematic product differences could be related with preparation methods rather than product (Sanchez & Chambers, 2015). Certain food carriers also impact on the flavour of the product under study (Cherdchu & Chambers, 2014); then for foods added with EOs carriers should be avoided. Besides, food preparation at home is different than food preparation in controlled conditions

used in studies with trained panelists, thus perception and attributes could be different between both products. For sensory analysis of foods added with EOs special care is needed in order to reduce false differences amongst products related to food preparation or sample preparation. It is important to adequately follow each procedure in order to be consistent with the selected methodology and ensure that obtained results are truthful and reliable. Preparation of foods is complex (cooking temperature, salt addition, additives, *etc.*); thus, when two foods are compared similar preparation conditions should be used. Similarly, serving temperature must be maintained equal for the two products in order to appropriately compare samples and characteristics of 'different' products. Temperature for meats is to be maintained at 50°C (in bath or oven) before serving and immediately given to panelists (Lawless & Heymann, 2010).

Sample preparation for olive oil containing oregano EO (as antioxidant and flavouring) consisted in pouring 14 ml of the sample in brown glasses identified with a 3 digit number code and covered for preserving flavour and aroma. One hour before the test all samples were heated at the same time (using a water bath) at $28 \pm 2^\circ\text{C}$ and let rest at room temperature ($20\text{--}25^\circ\text{C}$) in order for the head space to had enough time to develop (Asensio *et al.*, 2012). Ricotta cheese portions of 10 g were placed into cups (plastic), covered, and coded with random numbers; before sensory analysis, samples were let at room temperature ($20 \pm 2^\circ\text{C}$) for 1 h for their equilibration (Asensio *et al.*, 2014). In other studies, foods were mixed with the EO or taken from the storage stand and given to panelists, for example tomato paste with different EOs (Omidbeygi *et al.*, 2007), slices of mortadella with thyme and rosemary EOs (Giarratana *et al.*, 2016), roasted sunflower (3 g) with carvacrol or thymol (Quiroga *et al.*, 2015). For chicken fillets with oregano EO, were cooked (in microwave 5 min at full power, 700 W) and immediately presented to panelists, individual coded, on booths (Ntzimani *et al.*, 2010). For stored foods like poultry added with rosemary EO (0.2%), packaged in modified atmosphere, and stored at 4°C for seven days, poultry was removed from package and wrapped in aluminum foil, then individually cooked in an oven (at 220°C) for 20 min and served warm in a coded dish (Kahraman *et al.*, 2015). Other authors proposed to cook the meat before adding the EO. For minced beef meat added with three levels of *Salvia officinalis* or *Schimus molle* EOs, meat was steam-cooked during 1 h and after cooling (40°C), different lots were formed and mixed with 1.5, 2.0, or 3.0% of each EO. From each lot, meatballs (25 g) were formed and served on randomly coded containers (Hayouni *et al.*, 2008).

10.8 Sensory Analysis Methods

Essential oils can improve food flavour; however, if their application is as antimicrobial or antioxidant maybe undesirable tastes can be detected on foods. Thus, sensory analysis should focus in determining differences, the nature of the difference and if the difference is acceptable. If the EOs enhanced product, even higher level of flavouring could be consider. The main point of sensory analysis is defining product needs in order to conduct the right tests. In addition, the analyst should be clear what he/she wants to determine; overall difference, attribute difference, relative preference, acceptability, *etc.* Furthermore, the analyst should know how test results are to be interpreted and utilised. Regarding food quality sensory analysis of EOs is a simple, cheap and fast technique for identifying gross falsifications but not to identify more subtle adulterations

(Do *et al.*, 2015). Studies related to EOs addition or foods treated with EOs as antimicrobials or antioxidants, usually include sensory analysis in order to determine changes or undesirable tastes. Objective and subjective tests are commonly used. Objective tests are carried out by trained panels, meanwhile subjective tests, also known as affective or consumer tests, are carried out by untrained panelists.

10.9 Descriptive Tests

Descriptive sensory analysis include the most complete techniques that allow to obtain information about ingredient and process variables, sensory attributes that are important for acceptance and a complete sensory description of a product. In order to carry out the descriptive analysis between 8 and 12 trained panelist are usually required (Lawless & Heymann, 2010). Panelists have to be trained with standards as references in order to understand and identify specific attributes; generally, the intensity of attributes is related to a quantitative scale. These tests could be related with instrumental measurements and be highly reproducible and consistent if panelists were trained successfully. Thus, consumer panelists have to be avoided in these type of tests. Descriptors used for specific attribute have to be clearly defined in scientific language, but also in common language when tests will be related to consumer acceptance. A suitable delimitation and single descriptor for an attribute is desirable in order to avoid panelists' confusion or ambiguity.

Main descriptive analysis techniques include (a) Flavour Profile®(FP), a qualitative test, when a numerical scale from 1 to 7 points is included, the technique is renamed as Profile Attribute Analysis. These tests use specifically trained panelists according with product's attributes. Tests include a panel leader who is accountable for interaction within the panel, preparation of the samples and standards for reference, as well as encouraging the panel to a unanimous product description. (b) Quantitative Descriptive Analysis®(QDA), a quantitative test that utilises unstructured scales to define the intensity of assessed attributes. Panelists define the terms that portray differences amongst products and standardise the lexicon to describe them. QDA may be used for complete description of a narrow range of product's attributes from initial visual evaluation to aftertaste. Panelists are not influenced or persuaded to a consent product description by the panel leader. QDA is faster than FP, and data obtained by means of QDA can be statistically analysed by means of an analysis of variance. (c) Texture Profile® (TP) is a technique that allows the assessment of products texture characteristics, from the first bite through complete mastication. TP utilises a vocabulary (standardised) to describe textural features of any product; product-particular terms can be employed from the standardised terminology of a specific product. The full specific parameters are fixed by product, having the particular characteristics as major components. (d) Sensory Spectrum® test is a further extension of descriptive techniques; trained panelists should be absolutely calibrated. During test, different references have to be included to anchor specific levels of an attribute. The training of panelists is very intensive and costly; thus, this technique is frequently used by organisations with quality assurance regularly based on sensory analysis.

Some aromatic components of spices determine their quality and are closely related with their EOs. Sometimes sensory evaluation is a useful technique to determine spice

aroma quality and its degree of superiority when several cultivars are compared. Mamatha *et al.* (2008) used the QDA technique to determine the quality of EO of three cultivars of pepper. Odour analysis of pepper EO also was evaluated by the same QDA scale. Odour profiles were useful to differentiate and identify specific notes for each cultivar of pepper. Quantitative descriptive sensory analysis has been used in order to describe the profile or to identify specific characteristics of EOs; Périno-Issartier *et al.* (2013) conducted a sensory descriptive evaluation with 12 trained panelists with the purpose of identifying four descriptors in EO of lavandin flowers obtained by eight methods: hydrodistillation, steam distillation, turbo-hydrodistillation, ultrasound-assisted extraction followed by steam distillation, *in situ* microwave-generated hydrodistillation, microwave steam distillation, microwave hydrodiffusion and gravity and microwave steam diffusion. Descriptive adjectives were musk scent, flowered odour, herbaceous scent and camphor smell and were submitted to panelists, then marks were assigned according to the concordance between extract and descriptive adjective, a value of 0 indicated extract was not in accordance while 10 that the extract was in perfect accordance with the adjective. Musk smell in the EO extracted by turbo-hydrodistillation was considered the less-pleasant odour. Sensory findings were well related with the instrumental composition of EO. Similar reports are listed in Table 10.4. Adjective descriptors used for EOs are variable and depend on the specific source of EO; however, some descriptors are common on all studies as can be observed in Table 10.4.

Traditionally, EOs are used as flavourings in foods and beverages. Thus quality and sensory acceptability have to be evaluated. Camel's milk added with three levels (0.03, 0.06, or 0.09 ml/L) of spearmint (*Mentha spicata*) or wild thyme (*Zataria multiflora*) EOs in order to flavour milk and enhance its sensory attributes. A descriptive sensory test was applied to evaluate its organoleptic properties; the addition of spearmint EO (of 0.09 ml/L) enhanced the milk's sensory properties (Maaroufi *et al.*, 2015).

Several studies reported sensory analysis when EOs are added to food through direct, indirect, or semi-direct methods. Authors were able to identify changes in a specific attribute or the presence of specific undesirable taste by means of trained panelists. Table 10.5 presents some reports regarding sensory analysis of foods added with EOs by descriptive tests. Foods, purposes of the sensory analysis, number of trained panelists and results are variable; and sometimes researchers did not reach the minimum number of trained panelists required for the test. On the other hand, descriptive tests also assessed full characteristics of foods added with EOs, it means not only flavour and odour are evaluated but texture and colour are also included. In these cases, descriptive tests of flavour could be combined or hybridised with textural or SpectrumTM tests. These sensory tests provided useful detail information with regards to flavour, appearance, aroma and texture of a multi-component/ingredients food matrix. Further, in some studies these descriptions were related to instrumental, chemical or physical properties. Furthermore, if these tests were used to evaluate changes in the intensity of specific attributes over time, they were able to determine deterioration reactions and shelf-life, as well as when these attributes were unacceptable to consumers. The variety of plant from which EOs were obtained has an effect on sensory properties of food. EOs from four varieties of oregano, Compacto: *Origanum vulgare* L. ssp. *vulgare*, Cordobes: *Origanum vulgare* ssp. *hirtum* (Link), Criollo: *Origanum vulgare* ssp. *hirtum* (Link), or Mendocino: *Origanum x majoricum* Cambess; were tested at 0.05% (w/w) in ricotta cheese as antimicrobial and flavouring agents. Sensory evaluation was carried out by

Table 10.5 Sensory analysis of selected foods by means of descriptive tests.

Food	Application method of EO	Attribute	Purpose of the test	No. of trained panelists	References
Extra virgin olive oil	Direct addition of selected amounts of mint and thyme EOs	Flavour and odour	Evaluate the sensory response to flavoured olive oils	20	Moldao-Martins <i>et al.</i> , 2004
Tomato paste	Direct addition 500ppm of thyme, summer savory, and clove EOs	Flavour and odour	Identify differences amongst free paste or added with EOs	50	Omidbeygi <i>et al.</i> , 2007
Cooked coated chicken fillets	Direct addition as coating of 0.2% of rosemary EO	Taste and odour	Find the first off-odour or first off-taste during storage and the EO effect	7	Ntzimani <i>et al.</i> , 2010
Italian salami	Direct addition 0.005 or 0.010% of coriander EO	Unstructured scale of 10 cm for QDA technique	Sensory profile described through 12 descriptors, after 90 days of maturation	12	Marangoni & Moura, 2011
Cooked rainbow trout coated with chitosan + thyme EO	Semi-direct 0.1% of EOs in edible coating	Taste	Identify differences amongst three products	7	Chamanara <i>et al.</i> , 2012
Dates coated with chitosan + bergamot or bitter orange EO	Semi-direct 0.2% of EOs in edible coating	Sensory profile with 17 attributes	Evaluate citrus odour and flavour as well as other attributes	10	Aloui <i>et al.</i> , 2014
Camel's milk	Direct addition with 0.03, 0.06 or 0.09 ml of spearmint or wild thyme EO/L	Taste, aroma, colour and overall acceptability	Evaluate quality of Camel's milk	9	Maaroufi <i>et al.</i> , 2015
Cooked ground beef	Direct addition of combined EOs (0.0125 to 0.2%) of Chinese cinnamon and cinnamon bark)	Smell and taste	Find the highest sensory accepted concentration (0.05%)	10	Ghabraie <i>et al.</i> , 2016

EO = essential oil, QDA = quantitative descriptive analysis.

QDA and Spectrum™ with a panel of seven trained panelists. EOs from oregano Compacto displayed a substantial increase of surface moisture. Oregano EOs from Cordobes or Compacto were similar in sweetness intensity to cheese without oregano EO, meanwhile oregano EO from Cordobes had the lowest intensity in sourness. Nevertheless, a strong association was observed amongst oregano flavour, fermented, sourness, surface moisture, glossiness and sweetness with tested oregano EOs, thus had an important effect in ricotta cheese sensory properties (Asensio *et al.*, 2014). The same authors tested the same four oregano EOs from the same varieties in extra virgin olive oil in order to flavour it and retard its oxidation. Sensory analysis of the samples were realised by 126 days every 21 days in order to evaluate changes in positive attributes (fruity, pungent, bitterness and oregano flavour) and negative attribute (rancid) with 8 trained panelists using a line intensity scale (10 cm, where 0 is the lowest value and 10 the highest value). At the end of the study, oregano EO from Cordobes was the one that better preserved positive attributes (specially bitterness and pungency) in extra virgin olive oil due to its antioxidant activity (Asensio *et al.*, 2012). In this case, QDA test was used to determine undesirable flavour profile of food (olive oil) and then relate it with the effectiveness of the EO, when used as an antioxidant. The absence of undesirable flavour of food indicated an adequate antioxidant effect of EO. Table 10.6 presents some examples of adjective descriptors used to depict selected foods in sensory descriptive tests.

10.10 Discrimination Tests

Discrimination tests are used when minimal changes in two foods are expected. These tests are widely used in products with partial substitution of their ingredients or in products subjected to a new process. The main purpose is to know if the consumer is able to detect changes or differences between two products. Main discrimination tests are paired comparison, duo-trio, triangle, dual standard and A-not-A.

Foods added with EOs are strongly affected in their sensory attributes; thus, important changes are expected in food sensory attributes; thereby the use of sensory discrimination tests is common. Some authors have used these tests when minimal amounts of EOs are used or semi-direct addition of EOs through edible coating is studied. Duo-trio and directional paired comparison tests were performed for olive oil added with 0.05% of oregano EO after 126 days of storage at 23 °C in order to identify which sample contained oregano EO by means of 20 consumer panelists. Consumer panelists significantly ($p \leq 0.10$) detected the flavour of oregano EO when directional-paired comparison test was used. Results of the duo-trio discrimination test showed that panelists were not able to distinguish differences ($p > 0.05$) amongst olive oil with or without oregano EO (Asensio *et al.*, 2013).

Triangle tests were used to evaluate fillets of dried fish (3x2 cm) covered with whey protein edible film containing 4 or 6% of anise oil by spraying, packaged and stored at 30 °C for 24 h. Fillets were fried in vegetable oil for 1–2 min and served to 30 untrained panelists. For each test, panelists were presented with three samples; two of them edible coated without EO and one with edible coating containing 4 or 6% of anise EO, or the reverse. Fillets with edible coating contained 4% of anise EO were not significantly ($p > 0.05$) different from fillets with edible coating without EO, while fish fillets with edible coating added with 6% of anise EO were detected as different (Matan, 2012).

Table 10.6 Adjective descriptors used to describe sensory profiles of selected foods added with essential oils.

Food and added EO or compound	Application method	Adjective descriptors	Sensory quality	No. of panelists	References
Fried salted peanuts with 0.2 g/kg of oregano EO	Direct addition on surface through vegetal oil	Brown colour, roughness, glossiness, oregano EO aroma, olive oil aroma, roasted peanut oxidized, cardboard, sweetness, saltiness, sourness, bitterness, astringency, crunchiness, and hardness	Evaluates sensory stability of fried salted peanuts	12	Olmedo <i>et al.</i> , 2009
Extra virgin olive oil with 0.05% of oregano EO	Direct addition as antioxidant and flavouring	Positive attributes (fruity, pungent, bitterness, oregano flavour), and negative attribute (rancid)	Evaluate changes during storage in the intensities of positive and negative sensory attributes from extra virgin olive oil with the addition of oregano EOs from 4 varieties	8	Asensio <i>et al.</i> , 2012
Ricotta cheese with 0.05% of oregano EO from four different varieties	Direct addition as an ingredient	Appearance (colour, glossiness, surface moisture), aroma (cheese taste, cooked milk/ milky, and oregano flavour), taste (sweet, salty, bitter, and sour), aroma negative attributes (rancid milk cream, and vinegar)	Sensory properties of ricotta cheese flavoured with oregano EO	7	Asensio <i>et al.</i> , 2014
Fresh cut celery, leek and butternut squash sprayed with aqueous dispersion of tea tree EO (30 µL/ml)	Direct addition on surface	Overall visual quality included attributes visually perceived as freshness, surface brightness, uniformity of colour, and texture	5-cm unstructured intensity scale (0 = highly deteriorated aspect, 5 = fresh product)	9	Alvarez <i>et al.</i> , 2015
Roasted sunflower seeds with 0.2 g/kg of carvacrol, sabinene hydrate or thymol	Direct addition on surface through vegetal oil	Brown colour, roughness, glossiness, roasted sunflower flavour, oxidized, cardboard, carvacrol flavour, sabinene hydrate flavour, thymol flavour, bitterness, sourness, astringency, crunchiness, and hardness	Sensory stability of roasted sunflower seeds	10	Quiroga <i>et al.</i> , 2015
Poultry fillets added with 0.2% of rosemary EO and modified atmosphere packaging	Direct addition	Flavour intensity (flavour intensity, spicy taste, salty taste, sweet taste, and acidic taste), odour (odour intensity, sour odour, sweet odour, and spicy odour),	Evaluate sensory attributes	8	Kahraman <i>et al.</i> , 2015
Mortadella with 0.025% thyme EO and 0.025% of rosemary EO	Direct addition as an ingredient	Global appearance, slice surface, acid taste, fatness, hardness, juiciness, pleasantness, and typical mortadella aroma	Scale with 20 points (0 excellent mortadella, 20 not edible mortadella)	3	Giarratana <i>et al.</i> , 2016

EO = essential oil.

10.11 Time-Intensity Methods

Especially in the case of EOs, quantity and quality parameters of the active compounds (mainly used as flavourings, antimicrobials and/or antioxidants) are very important with regards to sensory characteristics and therefore for consumer acceptance. Every EO is distinguished by its intense and lingering odour, and these parameters are close related with active components' properties and quality (with respect to the volatile compounds). These properties can be sensory evaluated by time-intensity methods, registering variations in perception intensity with respect to time. There are three time-intensity methods: discrete or discontinuous sampling, 'continuous' tracking and temporal dominance techniques. These methods allow the use of descriptive panels (Lawless & Heymann, 2010).

A time intensity study was carried out to evaluate pepper EO quality for three different cultivars. Flavour intensity was measured with a 15 cm-structured QDA scale, 1.25 cm was marked as recognition threshold and 13.75 cm as saturation threshold. The group of trained panelists was integrated by 10–12 people. A 0.1% pepper EO dispersion in cornstarch gruel was served in 25-ml containers. Perceived sensation or onset of flavour sensation was marked by panelists as soon as they took the samples in their mouths. Intensity was marked after samples were hold 10s in their mouths. Remaining flavour intensity scores were noticeable after every 10s up to 60s. The time intensity profile was obtained (plotting mean scores versus time), which is an indicator of the spice quality (Mamatha *et al.*, 2008).

Cumin EOs of seeds from eight different regions of India were evaluated by a time intensity test to evaluate quality of the spice regarding to its volatile components. Aroma intensity was scored by panelists on a 15-cm structured-scale for quantitative descriptive analysis with anchoring at two levels, low (1.25 cm) and high (13.75 cm) with thresholds of 0.02 and 2.00% of cumin extracts, respectively. Panelists marked the intensity of cumin aroma on the scorecards starting from the onset of aroma and every 10s up to complete 60s. By means of a flavour intensity test, panelists' evaluated 1% cumin extract; each panelist received a 25-ml beaker with 10 ml of extract, panelists marked their perceived sensation as soon as the sample was in their mouth (without swallowing), and then panelists were requested to hold the sample in their mouth for 10s and then swallow it and grade the intensity. After swallowing, panelists continued grading the intensity every 10s up to 60s. Time intensity profiles were obtained by plotting average scores versus time (Ravi *et al.*, 2013).

10.12 Preference Tests

Preference tests are also known as consumer tests in which consumers indicate product most liked, usually from a pair. A final product may be evaluated by this type of tests to determine its acceptance considering its sensory attributes. This type of test is separated from analytical sensory techniques due to its affective nature. The test does not attempt to find or identify differences amongst products. Moreover, the acceptability information is very useful to know whether consumer expectations are met or if product formulation is appropriate. For simple paired preference testing, a minimal of 20 consumers should evaluate the samples for preference.

Several studies regarding EOs added to foods have used this type of test to measure acceptability of the formulated product. In some cases, the purpose of the test was to know which product the consumers most liked between added or not with EO. Other studies evaluated amongst three levels of added EO as flavouring and used a preference test in order to know which was the level preferred by consumers (Gonçalves-Cattelan *et al.*, 2015). Table 10.7 presents some studies that utilised preference tests. The number of consumers asked to evaluate the foods are wide variable (from 20 to 130) as well as the tested foods.

A paired preference test was used to assess acceptance of EO when added to foods; Du *et al.* (2012) used a paired preference test in order to evaluate the acceptability between two levels 0.5 and 0.75% of carvacrol and cinnamaldehyde (the active ingredients of oregano and cinnamon EOs, respectively), when they were added to tomato or apple films used to wrap chicken breasts. Consumers (52–63 individuals) had to choose the best taste between baked chicken that had been wrapped with films with or without carvacrol or cinnamaldehyde. Panelists preferred carvacrol-containing tomato-film-coated chicken over corresponding apple coating; meanwhile cinnamaldehyde-containing apple-coated chicken was preferred over corresponding carvacrol-containing wrap. Films containing 0.5% of carvacrol and cinnamaldehyde were not identified as different from films without them. Ranking classification or sensory ordering also has been used to determine threshold of EO detection; Valero and Salmerón (2003) applied a test of sensory ordering in order to evaluate sensory effects of different concentrations of cinnamon EO in carrot broth.

10.13 Sensory Analysis Reports

After sensory tests are applied data should be analysed. Techniques of statistical analysis are useful in order to summarise information and letting the sensory analyst to make wise conclusions from the experimental data. Three outcomes can be derived from statistical analysis, first simple description of the results such as averages and standard deviations. Second result is to provide evidence that the ingredient or process variable have an effect on the product sensory attributes, and change(s) are not a chance of variation. Thus inferences can support the conclusions about product and variables that were tested. A third result is to estimate correlations between experimental variables and the measured attributes which allows adding valuable information through equations and models generated from data (Lawless & Heymann, 2010).

Data from sensory evaluations can be reported in tables or graphs and then statistical analyses should be performed. Arithmetic mean, coefficient of variation, and standard deviation are calculated for data description. The confidence interval, statistical hypothesis, and statistics to compare means like student's t-statistic (for small experiments with observations ≤ 50) allow inference about data. For test analysis requiring categorised right and wrong answers, or counting the numbers of choosing one product over another (like triangle test and paired preference test), binomial distribution is useful. A multinomial distribution applies when more than two alternatives for classifying responses are possible. The common statistic for comparing two or more categorised responses is the chi-square statistic. Other statistical tests follow a chi-square distribution for independent observations are the McNemar test, Stuart-Maxwell test,

Table 10.7 Sensory analysis of selected foods by means of preference tests.

Food	Application method of EO or compound	Attribute or specific question	No. of consumers	References
Melon and watermelon juices	Direct addition 0.3% of cinnamon bark EO	Rate the preference of odour, colour and taste in a hedonic scale of 11 points	30	Mosqueda-Melgar <i>et al.</i> , 2008
Fresh cut iceberg lettuce, beet, and rocket	Direct addition by immersion (washed with solutions of 1.25 μ L/ml of oregano EO or 20 μ L/ml of rosemary EO)	Product acceptance using a 5 point hedonic scale and purchasing intention	50	de Azeredo <i>et al.</i> , 2011
Italian-type sausage with Olive oil	Direct addition by spraying on surface of basil EO (aqueous dispersions from 10 to 100 mg/ml)	Product taste acceptability	45	Saggiorato <i>et al.</i> , 2012
Roasted sunflower seeds	Direct addition of 0.05% of oregano EO	Evaluate odour and flavour acceptances using a 9-point hedonic scale	100	Asensio <i>et al.</i> , 2013
Whole red porgy (fish)	Direct addition on surface of 2% of oregano or poleo EO	Determine acceptability using a scale of 9 points	100	Quiroga <i>et al.</i> , 2013
Cakes	Semi-direct (coated with chitosan + oregano EO at 0.1%)	Score odour and taste of fish free or with chitosan-EO edible coating using a 0–5 acceptability scale	51	Vatavali <i>et al.</i> , 2013
Orange juice	Direct addition as an ingredient (0.18 g/kg of Ajwain, camel thorn or lemon balm EOs)	Rate how much they liked the taste using a hedonic scale of 9 points	20	Saatchi <i>et al.</i> , 2014
Salad dressings	Direct addition (50, 100 or 200 ppm of orange (<i>Citrus sinensis</i>) EO)	Determine the hedonic acceptance using a scale of 9 points	55	Espina <i>et al.</i> , 2014
Biscuits	Direct addition as an ingredient of 0.2 to 0.4% of oregano EO	Study the influence of EO and NaCl in sensory acceptability using hedonic scale of 9 points	60	Gonçalves-Cattelan <i>et al.</i> , 2015
Dry fermented sausage (chouriço)	Direct addition as an ingredient of different amounts of <i>Citrus aurantium</i> peels EO	Degree of acceptance of product's aroma	30	Trabelsi <i>et al.</i> , 2015
Soy sauce	Direct addition as an ingredient of 0.005 or 0.05% of different EOs	Evaluate consumption of chouriço with EO	104	García-Díez <i>et al.</i> , 2016
	Direct addition as an ingredient of 0.25, 0.5, 1.0 or 2.0 mM of carvacrol or thymol	Evaluate odour preference	30	Moon and Rhee, 2016

EO = essential oil.

beta-binomial, chance-corrected beta binomial and Dirichlet multinomial analyses. Rank order tests can be analysed with Mann-Whitney u-test when two samples are evaluated (also can be used for paired comparison), while for more than two samples Friedman and Kramer tests can be used (Lawless & Heymann, 2010).

Sensory analysis sometimes examines dissimilarities amongst numerous treatments or levels, to compare several means at the same time; in this case, analysis of variance (ANOVA) is a useful tool. In ANOVA, a ratio of the factor variance to the error variance it is constructed, and ratio follows the distribution of an F-statistic. Main types of ANOVA are complete block designs (when every panelist views every product or every level of the treatment) and multiple factor ANOVA (useful for analysis of two or more independent variables). The first step in ANOVA with more than two products is to find a significant F-ratio; then the comparison amongst means is necessary in order to know which pairs are different. Common techniques use to compare means after ANOVA are Scheffé, Tukey, Honestly-significant-difference (HSD), Newman-Keuls, Duncans and least-significant-difference (LSD). A useful design for sensory analysis is the two-way ANOVA with every panelist rating every product and replicated ratings, useful in descriptive designs in which the score depends on the treatment, judge, replication, interactions and error. Design of experiments is used in sensory analysis in order to generate an accurate and precise estimate of the experimental error, and these designs include random (complete) block designs, balance incomplete block designs, Latin-square designs and split-plot designs (Meilgaard *et al.*, 2007; Lawless & Heymann, 2010), as well as factorial designs and response surface methodology designs (Meilgaard *et al.*, 2007). Research can be focused on the effect of some factor or factors that have been applied to the samples, such as specific ingredients, in which situation specific designs that provide precise and complete comparisons of the effects of studied factors are needed, while at the same time minimising the total amount of experiments required to perform the study.

When scientists want to know if there is a significant association between two set of data, specifically independent and dependent variables, a coefficient of correlation can be established. However, correlation coefficients should be used carefully due to not direct relation between data and multivariable relations between data. Main correlation coefficients are: Pearson's correlation coefficient, coefficient of determination, linear regression, multiple linear regression and other measures of association (Spearman rank correlation and Cramér's V measure). In addition, preference mapping can be applied to relate sensory data and consumer acceptance. Internal preference mapping (use consumer acceptance ratings to construct the map), external preference mapping (use sensory descriptive attribute ratings to construct the map) and partial least-square regression (use both consumer and sensory data to locate the products on the map) are the major varieties of preference mapping utilised for sensory analysis (Meilgaard *et al.*, 2007; Lawless & Heymann, 2010).

Characterisation or descriptive profiles of EOs are generally reported in typical graphs that show main descriptors and their intensities, such as the one presented in Figure 10.2. Time intensity profiles were obtained by plotting mean scores versus time. Most studies of foods added with EOs show their descriptive tests or their preference tests results by numerical or graph data summarised as means \pm standard deviation with ANOVA at significance level of 5% and means' comparison using LDS, Fisher, Duncan's multiple range tests, or Tukey's test. These types of reports have been used

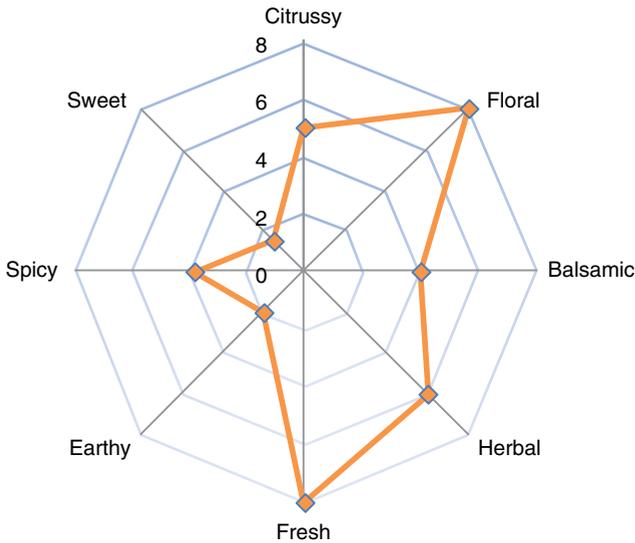


Figure 10.2 Descriptive sensory analysis of odour attributes from an essential oil.

to present attribute intensity ratings of fried-salted peanut added with oregano EO (Olmedo *et al.*, 2009), scores of descriptive terms for Italian salami added with coriander EO (Marangoni & Moura, 2011), scores of descriptive EVOO added with oregano EO (Asensio *et al.*, 2012), scores of descriptive roasted sunflower seed added with oregano EO (Quiroga *et al.*, 2013) or thymol, sabinene hydrate, orcarvacrol (Quiroga *et al.*, 2015), acceptability attributes of salad dressing added with oregano EO (Gonçalves-Cattelan *et al.*, 2015), scores of odour intensity compounds of orange EOs from six regions (Xiao *et al.*, 2016b), or scores of odour intensity of six attributes for five *Chrysanthemum* EOs (Xiao *et al.*, 2016b), scores of ground beef meat with selected concentration of combined EOs, Chinese cinnamon and cinnamon bark (Ghabraie *et al.*, 2016), preference scores for soy sauce added with carvacrol and thymol (Moon & Rhee, 2016). Correlations were also used for sensory descriptive attributes related to chemical composition of fried-salted peanuts utilising a Pearson correlation coefficient, a linear regression was applied to determine if time (independent variable) affected sensory attributes (Olmedo *et al.*, 2009). Pearson's correlation test was used to determine possible correlation between sensory attributes and formulations of salad dressings added with oregano EO (Gonçalves-Cattelan *et al.*, 2015).

A partial least squares regression was performed with compounds determined by GC-MS and sensory attributes in order to obtain the correlation between sensory attributes and orange or *Chrysanthemum* EOs odour active compounds (Xiao *et al.*, 2016a,b). Others used regression analysis from an experimental design to evaluate the influence of carvacrol, sabinene hydrate or thymol on some undesirable flavours of roasted sunflower seeds at different times of storage (Quiroga *et al.*, 2015). Regression analyses utilised to determine equations for dependent variables (fruity, pungency, bitterness, oregano flavour and rancid flavour) in EVOO added with oregano EO from a factorial design was used to estimate storage time effects by Asensio *et al.* (2012). Quality changes mathematical modelling was performed with a general equation from

a zero-order model and regression analysis for three vegetables (celery, leek, or butternut squash) treated with tea tree EO to model overall quality changes (visually) and estimate sensory shelf-life (Alvarez *et al.*, 2015).

10.14 New Approaches to Reduce Undesirable Sensory Effects of Essential Oils

Direct addition of EOs to foods leads to a faster reduction of microbial load but sensory properties can be modified up to alter the taste of food or exceed an acceptable flavour threshold. Some alternatives to reduce sensory impact are adding the EOs through systems of controlled delivery, such as antimicrobial active package (films added with EOs), encapsulated EOs, and edible coating added with EO. In addition, encapsulated EO limits the composition degradation/loss during processing and storage, while controlling the delivery of the compound at a desired time and site; therefore, encapsulation is favorable prior to EO use in food products (Beirao da Costa *et al.*, 2013). Besides, the use of multi-hurdle technologies when EOs are utilised as antimicrobials or antioxidants can reduce the necessary amounts of EOs to reach the desirable preservation function, while sensory attributes are acceptable. EOs have been combined with other preservatives and/or preservation processes like lysozyme, other EOs and vacuum packaging for preserving cooked chicken fillets (Ntzimani *et al.*, 2010).

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11

Global Regulation of Essential Oils

Ismail Es¹, Amin Mousavi Khaneghah² and Hamid Akbariirad³

¹ Department of Material and Bioprocess Engineering, Faculty of Chemical Engineering, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil

² Department of Food Science, Faculty of Food Engineering, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil

³ Department of Food Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran

11.1 Introduction

There are more than 7 billion people on earth in 2016. The world population can give us an idea to estimate an approximate number of consumers since each is considered a potential user of goods and services. According to the study conducted by United Nations and University of Washington (Gerland *et al.* 2014), with the 80% probability, the world human population highly likely will reach 9.6 billion in 2050, and peak up to 12.3 billion or more by the end of this century. In another statistical survey from 'IIASA's World Population Programme (POP)', the population will go up to 9.4 billion and fall below 9 billion by 2100. Each statistical data has its assumptions, and it is hard to estimate which survey has a more accurate prediction; however, along with escalating growth of world population, together with the obligation to find new food resources, the consciousness of consumers has also been continuously increasing. This increase resulted in significant demand for foods with better quality and control, which also puts forward the term '*food safety and regulation*' on a global scale.

Also, according to the monitoring activities of governments, the occurrence of foodborne disease has been increasing. With the consistent global incidence of these diseases, several trends in food systems, food safety and regulation are becoming an issue of growing importance and receiving, even more, worldwide attention.

It should be reminded that foodborne diseases are significant burden for countries since governments have a great responsibility in taking precaution to protect the population from microbiological, chemical and physical hazards in food supplies. This responsibility includes ensuring that consumers are conscious of the content of the food they buy. Thus, many legal regulations on food safety have to be established by governments for appropriate food regulation. However, depending on the development level of countries, different policies are being applied.

Food safety is an issue of tremendous importance and must be regulated delicately due to its vigorous relation with public health. Another critical point in food safety is

that varying approaches can be employed for its regulation and depend on the category of compounds included into the commercialised product; intensiveness of these approaches can widely vary. As a part of the content of this book, these regulations will be limited to the worldwide use of essential oils.

Essential oils exhibit immense diversity regarding chemical functions; therefore, are widely used for industrial applications. However, essential oils contain compounds with undesirable properties, which, in most cases, can be unpredictably hazardous, in this context new cautions have been taken by international authorities (Do *et al.*, 2015).

Before citing global institutions involved in essential oil safety and regulation, it is important to highlight the difference between the terms 'law and regulation' in order to understand how governments and related organisations treat this critical issue.

The rules have been governing our lives for centuries, and they are mostly imposed by morality and customs that play a major role in our lives. However, there are some rules called 'laws' which are made by governments. Laws exist because most of the time people do not live in a well-structured society in harmony with others. According to the Organisation of American States, laws are set of rules that provide a way to resolve in a peaceful manner all the disputes between individuals who have disagreements and conflicts. The regulation can be treated as if they are 'law' since they are set of rules or norms adopted by governments and strongly supported with penalties. The regulations can aim at non-profit organisations, other governmental institutions and even individuals. They can also be considered constitutions, statutes, legislation or standards. As well as laws, regulations seek to change behaviours in order to produce desired outcomes.

Regardless of the difference between legislation and regulation, the first question that comes to minds is: Is it exceptionally necessary to have a rigorous global control mechanism for the use of essential oils? The answer is obviously yes. Most of the time, people have the common view of that essential oils or similar ingredients are safe since they come from a plant source. However, most people are unaware of that many plants can also consist of compounds that are toxic, irritating or cause allergic reactions. Cumin oil, which is a valuable essential oil, can be given as an example. Cumin oil is considered safe in foods, but cause a blister on skin. Therefore, its use for the medicinal purpose is limited. Another example is the citrus oil, which is also safe in foods, however, exhibits side effects when used in cosmetics. The list can be easily extended. This suspicious behaviour of essential oils demonstrates the necessity of the existence of global institutions as a control mechanism.

11.2 Global Institutions Involved in Essential Oil Regulation

International food trade has been existing for centuries. Traditional knowledge on food trade had based on trial and error, which assisted people to know more about what to/not to eat. Most of the time, consumers buy foods that they have never touched or smelled and from producers that they have never seen. Therefore, there is a definite need for laws or regulations that could provide safety and quality in food regarding its production, transport, and trade to consolidate the confidence of consumers during this business. In the course of time, a global chain involving food trade related with the health of millions of people was born. These food laws exist in many countries and in

order to make sure that every nation benefits from these statutes, numerous global organisations started intense activities.

Essential oils are considered must-have substances of modern food products. The essential oils have existed for centuries and used as an additive in many products without the existence of a control mechanism. However, after the improved extraction methods and the proper use of essential oils in foods, they became an important part of the products that have been globally traded for years. Because of their possible adverse effects, laws and regulations to control the use of essential oils became a significant responsibility for these lawmaker organisations.

Following organisations mentioned below have been working firmly to regulate the safety of these vital substances that are part of our daily lives. It must be highlighted that the mentioned products in this chapter mostly contain essential oils at any concentration.

11.2.1 World Health Organisation (WHO)

During the United Nations Conference from 25 April to 26 June in 1945, San Francisco, for the first time, the proposal of establishing an international organisation, which would be responsible for all fields of public health, was submitted by the delegations of Brazil and China. For the first time, this organisation was established in 1946 as a specialised agency of the United Nations and named as World Health Organisation (WHO). WHO, which celebrated its seventieth anniversary in 2017, remains one of the oldest and most popular (in the public's mind) international organisations in the world.

Essential oil safety and its regulation must be considered a shared responsibility. The WHO reports all the findings that are obtained after intense global research. This valuable information underscores mostly the global threats caused by foodborne diseases and stimulates the governments, the food industry and consumers to collaborate and work hard to make food safer for prevention from these diseases. Therefore, food producers, suppliers, handlers and the general public need to be educated and trained on the prevention of foodborne illness. The WHO works closely with governments from all around the world (the region of Africa, South-East Asia, the Americas, Europe, Eastern Mediterranean and Western Pacific) to implement essential oil safety strategies and policies that will show a positive impact on the safety of these compounds at the global level.

WHO represents intense activity on International Food Safety Authorities Network (INFOSAN) that was developed by the Food and Agriculture Organisation of the United Nations (FAO). The publications on this network aim to strengthen global food control systems and ensure the information is shared rapidly and securely during emergencies to increase awareness of consumers. WHO built a strategic plan on World Health Assembly resolution WHA63.3 (May 2010) in order to provide a coherent framework by having precautions on priority issues that concern food safety and foodborne zoonoses during the period between 2013 and 2022. This plan was developed by the cross-sectoral collaboration of different food safety experts on global, regional and country level of WHO to adapt strategies and resolutions on food safety.

The WHO has an online portal (WHO Essential Medicines and Health Products Information Portal) that is responsible for the safety of medicinal plant materials, which some of them contain essential oils. The first global quality control methods for these

plant materials were published in 1998 in order to support nations in establishing quality standards and specifications for herbal materials. In 2011, an updated edition of these control methods was prepared to adapt to renewed national legislation and norms. In this publication, the WHO included chapters that cover the principles of ensuring the food safety and quality of herbal medicines, criteria to identify possible contaminants, techniques and reasonable procedures. The portal contains 5480 medicines and health products (data obtained in August 2016), and its database is updated monthly. A powerful search engine allows users to identify the related documents easily. The portal can be accessed at the following link (<http://apps.who.int/medicinedocs/en>).

There is a rapid increase in the use of herbal medicines, which contain important essential oils. The global market of these medicines has been expanding; therefore, the safety, efficacy and quality of herbal materials have become a major concern for international organisations, which participate this market growth. Due to complications of quality control, the latest World Health Assembly resolution (WHA56.31) on herbal medicines requested WHO to contribute technical support by developing a methodology to monitor this market and ensure the safety of these products. For this purpose, WHO published several guidelines on Good Manufacturing Practises (GMP) for herbal medicines. In 1996, WHO reported 'Good manufacturing practises: supplementary guidelines for the manufacture of herbal medicinal products.' Unfortunately, at that time, pressing concerns of GMP for herbal medicines were not considered priority by the member states. Subsequently, these concerns were discussed later on in details with a high priority while the worldwide use of herbal medicines was increasing. Consequently, more member states have started to set up their own national GMP for these medicines. WHO updated GMP guidelines (Annex 4 of WHO Technical Report Series, No. 908, 2003) and concluded them by 2005. These updated guidelines were accepted by a WHO Expert Committee in 2006 and published as Annex 3 of WHO Technical Report Series, No. 937.

Regarding the use of essential oil, WHO published 'The WHO monographs on selected medicinal plants' with four volumes (1999–2009) in order to provide scientific information on the safety, efficacy and quality control of these plants, which mostly contain essential oils. These monographs served as models to assist the member states from all around the world in developing their monographs for controlled use of essential oils.

An important detail about essential oil regulation is that EOs are not classified as medications and adverse events are not reported under the usual 'pharmacovigilance' reporting system initiated by the WHO. Consequently, this situation leads to a less efficient quality control of essential oils and related products.

11.2.2 Food and Agriculture Organisation of the United Nations (FAO)

A great need for an international organisation for food and agriculture emerged in the early 2000s. The president of United States of America, Franklin D. Roosevelt, reunited the United Nations (UN) in a conference in 1943 in order to discuss this common need. Forty-four governments from UN met in Hot Springs, Virginia, the United States from 18 May to 3 June with a view to establish a permanent organisation for food and agriculture. Two years later, the first session of FAO Conference took place in Quebec City, Canada and organisation committee established FAO as a specialised UN agency. FAO

headquarters then were moved to Rome, Italy from Washington, DC, the United States in 1951 and remain same so far.

FAO is composed of six departments, and each department focuses on updating policies to adapt to a global level to help eliminate food insecurity, which is an important issue since there have been still unclarified questions about the use of essential oils. FAO has been continuing its activities in strong collaboration with WHO. One of the most important outcomes was establishing Joint FAO/WHO Expert Committee on Food Additives (JECFA). JECFA is an international scientific expert committee (generally scientists with chemical expertise elected each five years) administered by FAO and WHO since 1956. This committee was established to evaluate the safety level of additives (processing aids and flavouring agents), contaminants or naturally occurring toxicants used in food products by aiming a better food safety regulation. JECFA completed safety evaluation of flavourings and their specifications, which is available from the WHO JECFA website, through the link Database of the assessment summaries. Specifications for flavouring substance, which mostly consists of essential oils, can also be found in a searchable online database at the FAO JECFA website.

Essential oil is an important issue for FAO. According to the reports published by FAO, because of the food spoilage and deterioration of food quality leads to the loss of 10–40% of all food produced all around the world and amongst all types of highest loss from fruit and vegetable crops that contain essential oils (Gustavsson *et al.*, 2011). Because of the importance and frequency of the use of EOs, FAO takes action to prevent this significant loss.

FAO released a report in 2005 that shows post-harvest operations of herbs, spices and essential oils in developing countries. In this report, a detailed guide to post-production operations such as harvesting, transport, threshing, drying, cleaning, packaging, storage and processing was given. Besides these operations, economic and social considerations at the global level were emphasised. In this report, many essential oils such as corn mint, Eucalyptus cineole-type, citronella, clover leaf, and so on, which have been regulated by FAO and their total global imports, can be found.

FAO has initiated a publication series on non-wood forest products, and one of the first publication series was in 1993 and 1995 focused on flavours and fragrances of plant origin. In this publication, many information about essential oil, mostly about their use, global demand and supply, trade conditions, quality criteria, the material of origin, methods of harvesting and processing and the development potential of the essential oil could be found.

According to this report, there have been many initiatives in the whole world, especially in developing countries, in order to establish new essential oil industries controlled by legislations. Although, the number of failures exceeded the successes most of the time because of several reasons and one of the reasons behind this failure was the lack of global regulation system. However, it must be kept in mind that instead of enforced regulation by legislation, alternative sustainable options must be put forward.

FAO also reported a great collection of 40 years through a publication called Food and Agricultural Legislation (FAOLEX). In this publication, FAOLEX provides information about treaties, laws, and regulations on food, agriculture and plant resources from all over the world. These materials come from the official gazettes sent by FAO's Member Nations in accordance with Article XI of the FAO Constitution. In the website

of FAOLEX (<http://www.fao.org/faolex>), it can be found regulations and policies related to essential oils (*i.e.*, Greek Ministerial Decree No. 1100/87, Hungarian Decree No. 89 of the Ministry of Agriculture on the production and distribution of seeds).

11.2.3 FAO/WHO Codex Alimentarius Commission (CAC)

Codex Alimentarius Commission (CAC) is an international organisation, which covers 99% of the world's population, established as an execution department of the Joint FAO/WHO Food Standards Programme in 1962. Its name comes from Latin, meaning Food (Alimentarius) and Law (Codex). After establishment, CAC held its first session in 1963 to bring regulation to global food standards. CAC is responsible for a great variety of collection of international food standards, guidelines and codes with the main purpose to increase the quality of consumers' health and ensure safe and fair food trade globally. In a meeting of CAC, the most significant issues discussed are mostly biotechnology, pesticides, food additives and contaminants. All related international food standards, which are a reference to nations, are available at <http://www.codexalimentarius.org>.

Especially, after the establishment of the World Trade Organisation (WTO), Codex standards have become a respectable reference for food safety under the WTO's Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement). The WTO Agreement on Technical Barriers to Trade (TBT) recognised these Codex texts as international standards, and TBT aims to ensure that the technical regulations, standards, and related procedures will not be discriminated or cause an unnecessary obstacle to international trade.

Methods of analysis and sampling of essential oils can be found in Codex General Standard for Fruit Juices and Nectars (Codex Stan 247-2005). In this stated document, essential oils are analysed and defined with specific code 'AOAC 968.20, IFU Method No. 45b'. CAC published another report in 2008 with the title of 'Guidelines for the Use of Flavourings' and coded as CAC/GL 66-2008. This guideline provides information for the safe use of the flavouring agents, which are evaluated by the JECFA. The scope of the guidelines is to determine that flavouring agents, whose corresponding specifications have been established by CODEX, should not present a safety concern at estimated levels of intake. Additionally, risk management, hygiene, labelling and other specifications of essential oils can be found in this report.

Ensuring the quality standards of essential oils and related products are reported as a major problem for the industry by FAO. These standards can be maintained by processing clean and high-quality products that have no contamination. In this case, the growers, processors, and traders have to take responsibility to ensure the hygiene requirements of products. CAC adopted Hazard Analysis and Critical Control Point (HACCP) that contains two sections that provide general guidance for food operation.

11.2.4 International Organisation for Standardisation (ISO)

ISO is an independent, non-governmental international organisation founded in 1946 by a small group of individuals (mostly engineers) and started its operational activities in 1947. ISO's headquarter located in Geneva, Switzerland and works with 162 national bodies. ISO has been bridging public and private sectors by innovating new high-level standards to provide solutions to business, government, and society.

ISO has representatives on various technical committees from member countries to set down internationally recognised standards. The relevant technical committee for essential oils is ISO technical committee 54, which have representatives from approximately 30 countries (Hunter, 2009).

Standard specifications for essential oils contain physical and chemical constraints such as refractive index, specific gravity, optical rotation, non-volatile residue, chemical specifications and ranges such as the level and range of a particular constituent in an essential oil based on a nominated method, acid values, ester values after acetylation, carboxyl and phenol contents may also be nominated depending upon relevance to the specific essential oil (Hunter, 2009).

For the first time, the definition of the term 'essential oils and related fragrance/aromatic substances' is mentioned in the ISO Norm 9235 Aromatic Natural Raw Materials in Geneva in 1997. In this ISO Norm, definition of essential oil is as follows: 'An essential oil is a product made by distillation with either water or steam or by mechanical processing of citrus rinds or by dry distillation of natural materials. Following the distillation, the essential oil is physically separated from the water phase.'

The organisation has developed more than 20,500 international standards and related documents for almost every industry from 1947 to 2015. ISO published these standards with 218 technical committees (TCs) worldwide. Amongst these TCs, the committee that is responsible for all essential oils is TC 54 to make essential oils more consistent. Their responsibilities include packaging, conditioning, and storage (ISO/TR210:1999), sampling (ISO212:1973), determination of optical rotation (ISO592:1998) and composition of oils. ISO codifies composition of each essential oil with an individual identification; for example, oil of rosemary (*Rosmarinus officinalis*) with ISO1342:1988; oil of basil (*Ocimum basilicum*) with ISO11043:1998. These codifications are mostly for food, pharmaceutical and cosmetic industry in order to ensure the identification of product and aim to expand global trade.

In ISO/TC 54, ISO also standardised many analytical methods to control the quality of these methods and specifications together with requirements for transport, labelling, and marking. ISO/TC 54 focuses on global trade in essential oils; consequently, improve the quality of essential oil market and protect the health of consumers who buy products containing essential oils and increase the safety level of these oils. Because of possible toxicity and hazardous effect of essential oils for pregnancy or people with asthma or epilepsy, this committee also participated in the revision of European Pharmacopoeia monographs.

11.2.5 Food and Drug Administration (FDA)

The U.S. Food and Drug Administration is a strong public health agency with scientific and regulatory functions on foods and drugs that have been developed in all around the world mostly in the United States of America. FDA is the oldest agency that has been established in order to protect consumers in the U.S. federal government. The idea of the foundation of such an organisation can be tracked back to the meeting set in 1820 to establish the U.S. Pharmacopoeia to bring standards to the use of drugs. After the foundation of the Department of Agriculture in 1862, the agency started its activities by establishing the Bureau of Chemistry, which was the predecessor of the FDA. Until 1930, the agency was known as the 'Food, Drug and Insecticide

Administration'. This name was shortened to FDA under an agricultural appropriations act after this date.

The FDA reviews applications for new drug and food products to determine whether these commodities provide sufficient evidence to be safe and efficient for the specified use. The process of new product approval by FDA begins after a producer submits an application after an extended period of research and development. During preliminary review, FDA determines whether the application is suitable to be reviewed. Depending on the potential of the product, FDA applies for either standard or priority review. Then, a specific commission of reviewers formed from medical officers, chemists, statisticians, microbiologists, pharmacologists and other experts from related fields is assigned by FDA for a more detailed analysis. This commission assesses an overall evaluation of safety, efficacy, design and quality of the studies performed during the production of drug or food. In the end, this review team either approve or cancel the application. The process of evaluation used to take very long time; however, FDA has decreased the average analysis time for a novel drug composition to six months, and in some cases, the drug has been approved even in less than six months.

FDA reviews essential oil and its substances under either cosmetic or drug category depending on the intention of the use of essential oil. If an essential oil is used in drugs, the regulation on the safety and quality of the essential oils more rigorous than evaluation of essential oil in a food product. Nevertheless, the essential oil is regulated under the cosmetic or food category as a flavouring agent or dietary supplements.

- Drug
...Known as a product which is used for curing or treating and effects on body functions.
- Cosmetics
...Known as a product which is used for body beautifying, cleansing and attractiveness.
- Dietary supplement
...Known as an ingredient or a product which contains minerals, herbs, vitamins or ... (FDA, 2016).

A wide range of essential oil components as a flavouring agent in food products have been accepted by FDA. Some of these substances are linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, ginger, tea tree, lavender, bay laurel, fennel and limonene. Beside these flavouring agents, the use of some crude essential oils such as clove, oregano, thyme, nutmeg, basil, mustard and cinnamon are also registered and classified as generally recognised as safe (GRAS) by FDA. The use of these essential oils and their components has to be approved by FDA before adding into food products. After the review process, FDA decides whether the essential oil meets the limit of accepted daily intake. The reason behind this rigorous and time-consuming regulation process of FDA is that essential oils can represent properties such as varying antimicrobial activities. For example, cilantro oil has sufficient antibacterial activity only at 0.018% *in vitro*; however, when applied to a ham model, cilantro oil shows no antibacterial activity even at 6% concentrations. Therefore, FDA has to investigate the effect of these oils by applying several tests before approval.

There are many chemical compositions that have been forbidden by agencies like FDA. One of them is phthalate esters, which is known as 'phthalates' and is a major

group of plasticising agents. They can be found in essential oils as contaminants or adulterants. This agent was banned in the European Union since 1999. In 2002, FDA considered this agent as a potentially toxic and advised that alternatives to phthalates should be used to keep plastics soft.

The FDA does not require approval for a fragrance that might contain essential oil since these fragrances are considered as cosmetic ingredients. Comparing to drugs and food products, the regulation process of FDA for essential oils that are used as fragrance ingredients is not that rigorous. Therefore, cosmetic manufacturer has significant responsibility to ensure the product quality. The FDA always requires a list of ingredients for products; however, it will not obligate the manufacturer to pass cosmetic secrets since the FDA does not have authority to require allergen labelling for cosmetics. This situation might bring disadvantage to the consumers especially to those with less knowledge of essential oils. The educated consumer will know what to/not to consume; nevertheless, it will be harder for the consumers who are new to essential oils.

FDA created Safety Information and Adverse Event Reporting Programme, which can be found at <http://www.fda.gov/Safety/MedWatch>. Consumers can report serious problems related to drug, food or cosmetic material. In case consumer experiences an adverse reaction to a cosmetic that might include essential oil, it can be reported the problem on this portal or calling 1-800-332-1088.

11.2.6 EU Commission

EU Commission and regulatory authorities in the EU consider essential oils 'biologically active substances'. For this reason, the commission demands the application of maximum precaution, which, consequently, will limit the use of herbs and spices added to foods. The commission has also defined security level for certain substances that are used in foodstuffs and beverages. For example, agaric acid 20, coumarin 2, santonin 0.1, hypericin 0.1, quassine 5 mg/kg (Baser & Buchbauer, 2010). For the past few years, strict regulations and directives have been developed by EU commission. These regulations already started to have impacts on the global trade of essential oils, particularly on labelling, which was discussed in the Seventh Amendment of the Cosmetic Directive. However, this situation will most probably change the behaviour of consumers since they prefer not to buy products labelled with potentially allergenic ingredients (Baser & Buchbauer, 2010).

11.2.7 United States Department of Agriculture (USDA)

At first, it should be mentioned that in FDA regulatory there is no group to be categorised as organic. Organic is a term that is used with marketing essential oils. The term 'organic' is not regulated by the FDA but by the USDA through the National Organic Programme (NOP). 'Organic' seems to have a fluid definition within our society. Many believe that it means that the product is free of chemicals and is safe.

The USDA definition for 'organic' is geared more for food, animal feed and fibre products. The grey area for this regulation is cosmetics/personal care products. This can include essential oil when they are marketed as a cosmetic and personal care products containing an essential oil (FDA, 2016).

11.2.8 Essential Oil Association of the United States (EOA)

The association was founded in 1927 and started its activity by serving the interest of processors, importers and dealers of essential oils and similar products in the United States. The Scientific Committee of the EOA, between 1940 and 1975, showed intense activities in the United States in order to develop specifications and test methods of essential oils. In this period, the committee developed over 300 specifications with 20 test methods to aid in the determination of essential oils. The report published by EOA, which was internationally accepted, contained all specifications as the legal part of EOA Standards and Specifications. These specifications filled the need that has not been covered previously by the United States Pharmacopeia (USP) and the National Formulary (NF). The Instrumental Analysis Committee had the mission to evaluate new spectroscopic methods to increase the quality of control methods.

11.2.9 Australian Regulatory

Like the other organisations, Australian food law agencies established some legislation about the essential oils. For instance, cosmetic claims are applied by National Industrial Chemicals Notification and Assessment Scheme (NICNAS) and NICNAS sets related laws on it and also about therapeutic goods (ARGCM, 2016).

11.2.10 Canadian Food and Drug Regulation

About the essential oil or essence or flavour, the Canadian food and drug administration regulates as follow:

‘It is derived from the plant which is aromatic, and it may contain preservative, food colour, sweetening agent and/or emulsifying agent and also it contains a kind of liquid such as water, any combination of benzyl alcohol, ethyl acetate, glycerol, glyceryl triacetate or edible vegetable oil’ (Canadian Food and Drug Regulations, 2016).

11.2.11 The American Essential Oil Trade Association (AEOTA)

The AEOTA was officially launched on November 1, 2014, and as a new non-profit business association, they aim to promote the safe and legal sale of essential oils and aromatherapy products in the United States. AEOTA also has a mission to work to influence the other regulatory organisations that have most of the control over essential oil and aromatherapy products by demonstration their ability to increase the quality of ethics, safety, and integrity of essential oils. More information can be found at following website (<http://aeota.org>).

11.2.12 The International Federation of Essential Oils and Aroma Trades (IFEOAT)

IFEOAT is an international organisation that favours the global flavour and fragrance market by supporting every organisation (from small family operations to large industrial) that does business in essential oils. IFEOAT was founded in London in 1976 when Ron Neal reunited a group of traders and users of essential oils in order to form an

international organisation that could represent this market. IFEOAT now has a membership of 450 companies representing more than 40 countries.

IFEOAT organises an annual conference, which will cover the topics of essential oils, aroma chemicals, fragrances and perfumes and cosmetics worldwide to bring together industry leaders. The last conference of IFEOAT was organised on 27 September at the Cinnamon Grand Colombo in Colombo, Sri Lanka. IFEOAT takes great responsibilities by validating numerous programme developed by different global institutions such as International Centre for Aroma Trades Studies (ICATS) and approves the membership of several centres by having strong international collaboration, the encouragement of good practise, the dissemination of information and provision of forums for discussion.

11.3 Conclusion

The use of essential oils in modern food, cosmetic and pharmaceutical products has become an important application since they significantly increase the quality of products. Moreover, due to the rapid growth of interest in these commodities, the concerns related to consumer health should be taken into consideration as an urgent issue. Hence, the establishment of organisations that act as a control mechanism for this problem becomes an obligation. The presence of organisations such as WHO, FAO, ISO or FDA has already helped the essential oil market to be well controlled in global level. However, this control mechanism should be reduced to national level since each nation has its specific consumer society, in this manner the control mechanism could be provided more efficiently. Also, the new discoveries of the potential use of essential oils in different products will keep increasing, and in order to avoid the possible hazard, essential oil regulation in both global and national level should constantly be renewed.

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12

Safety Evaluation of Essential Oils

Ramadasan Kuttan and Vijayasteltar B. Liju

Department of Biochemistry, Amala Cancer Research Centre, Kerala, India

12.1 Introduction

Essential oils are defined as complex, secondary metabolites which are volatile in nature and are extracted from the various parts of plants by the process of steam distillation or hydrodistillation and recently by supercritical extraction (Rubiolo *et al.*, 2010). Essential oils are used extensively in Asian and European countries. The key characteristics of essential oils are aromatic, volatile, liquid, lipophilic, coloured or colourless and generally with lower density than water and are enriched with biologically active constituents. They are produced by various plant parts like root, stem, bark, wood, leaf, buds, flowers and seeds and are stored in secretory cells, glandular trichomes, cavities and canals (Tongnuanchan *et al.*, 2014). Essential oils and their components are now gaining worldwide acceptance because of their potential multipurpose functional uses (Sawamura, 2000).

Essential oils (EOs) play a pivotal role in attracting the insects which in turn promote pollination, seed dispersal and maintain ecological balance. Essential oils in plants have also a role in the protection against pathogens and also they can act as insect repellent agents. The ingredients present in essential oils may be fluctuating based on climatic changes, nature of the soil, plant part, age, reproductive and vegetative stages (Masotti *et al.*, 2003; Angioni *et al.*, 2006). More than 3000 essential oils are known. Amongst these, 300 are widely used in pharmaceutical purposes, agriculture, food preservative and additives, sanitary products, cosmetic items and perfume industries and proved to be commercially significant (Perry *et al.*, 2003). Essential oils contain several chemical constituents, and this complex combination of compounds like phenols, sesquiterpenes, aldehydes, ketones and carbohydrates give them the characteristic fragrance, flavour and biological activities (Liju *et al.*, 2015; Bagora *et al.*, 2014). The chemical constituents present in essential oils shows the properties such as antibacterial, antifungal, antiviral, insecticidal and herbicidal with negligible after effects on host humans and animals (Sokmen *et al.*, 1999; Sumonrat *et al.*, 2008). EOs isolated from plants have been used for treatment of infectious diseases from the time immemorial (Sokmen *et al.*, 1999; Janssen *et al.*, 1993). Their potential for the prevention

and inhibition of malignancy progression has been investigated in recently (Zu *et al.*, 2010; Liju *et al.*, 2014a,b).

The use of essential oils is growing rapidly amongst most of the countries in food industries, production of soaps, detergents, cosmetics, nonalcoholic beverages, oral care products, aromatherapy and pharmacology (Buchbauer, 2000). Toxic effect of some essential oils may be due to their high concentration and effective permeability through lipid membranes as they are lipophilic in *nature*. However, most of the essential oils have acquired GRAS (generally recognised as safe) status from Flavour and Extract Manufacturers Association (FEMA) and got approved by the U.S. Food and Drug Administration (FDA) for safety food use. Even though the essential oils are safe, sometimes it may be harmful if not used carefully because they are highly concentrated secondary metabolites of plant parts. However, toxicity evaluations of essential oils are essential for their safe use to mankind. It will give more advantages to bring out the economic, scientific and traditional values of essential oils. This chapter mainly deals with the toxicological aspects of essential oils.

12.1.1 Use and Applications of Essential Oils

Nearly 100 essential oils now known are used as flavouring substances in food and non-food items. Essential oils gained wide range recognition as a multi-functional agent due to their strong aromatic effects and olfactory senses. Flavours and fragrances present in essential oils, directly act on the sensory receptor in mouth and nose give fine taste and smell. Essential oils are extensively used in beverages, baked foods, puddings, meat products, detergents, creams, lotions, perfumes and soaps. They are well known for their bactericidal, virucidal and fungicidal properties and they can act as an antiseptic agent (Irkin & Korukluoglu, 2009; Lee *et al.*, 2014). Moreover, they are notable for their anesthetic, sedative, analgesic and anti-inflammatory actions. Besides, essential oils are reported to exhibit antioxidant, antinociceptive, appetite stimulant, olfactory stimulant and insect repellent properties, which strongly indicate their biological activities (Misharina *et al.*, 2015; Ou *et al.*, 2014; Jeena *et al.*, 2013; Liju *et al.*, 2011; Kapoor *et al.*, 2009; George *et al.*, 2009; Irkin & Korukluoglu, 2009; Amer & Mehlhorn, 2006). They can reduce the level of nicotine craving in humans (Cordell & Buckle, 2013). Some of the essential oils can improve reflexive swallowing movement and have relaxant effects on the tracheal smooth muscles in humans (Ebihara *et al.*, 2006). Furthermore, their anticancer activity, chemopreventive effects and capability to inhibit aflatoxin induced DNA adduct formation have also been revealed recently (Liju *et al.*, 2014a,b; Jeena *et al.*, 2014; Hashim *et al.*, 1994). Moreover, EOs showed mortality against larvae of mosquito species and insects such as *Sitophilus oryzae* and *Corcyra cephalonica* (Amer & Mehlhorn, 2006). The biological activities of different chemical constituents present in essential oils have also been recently known.

12.2 Essential Oils and General Safety

Long history of the usage of essential oils in preservatives, food additives and traditional toxicology approaches has been pointed out to establish the safety of the EOs. Some essential oils which are mixed with considerable volumes of various diluents and chemicals may cause toxicity to healthy body and skin due to the presence of these added

components. Moreover, essential oils should be kept in brown bottles and kept away from light and heat to prevent oxidation of constituents present in it. Various animal studies showed that essential oils and their chemical constituents have a LD50 range from 1 to 20 g/kg body weight and some exceptions are also observed. Essential oil from *Salvia lavandulifolia* showed teratogenicity because this oil can produce an abortifacient effect. Some other essential oils showed oestrogenic and carcinogenic effects (Lis-Balchin, 2006). At times, essential oils that had not been tested on animals are sold by reputable dealers. This disregards the Trades Description Act (United Kingdom) and also manipulates with the human health and safety regulations. It is very important that essential oils which got tested on animals can only be sold legally (Lis-Balchin, 2006).

12.3 Safety of Essential Oils Used in Cosmetics and Industrial Applications

Nowadays the use of essential oils are found in many products like antiseptics, liniments, soaps, deodorant, flavours and cosmetic products. There is definitely a need to conduct extensive research and check on their toxicity. It is necessary to carry out toxicity evaluations before prescribing them for human use either internally or externally. Most of the essential oils play an important role in the field of cosmetics, food products, fumigants, detergents, perfumery, indoor and outdoor sprays, fibres and textiles (Rehman *et al.*, 2014).

Tea tree essential oil can be utilised in the formulations like of hand washes and antiseptics for the prevention of transmission of pathogens in the surgical environment (Messenger *et al.*, 2005). In the present era, antiseptics are used for post-surgical infections in buccal cavity. Essential oils from menthol, thyme, and eucalyptol can be used as mouth wash or antiseptic agents to prevent infections during oral surgery (Osso & Kanani 2013; Cavalca *et al.*, 2009). Generally, human beings use essential oils isolated from plants as means of protection from the mosquitoes as they are considered to be environment friendly unlike the synthetic chemicals. Essential oils from *Eucalyptus* species, *Ocimum* species and *Cymbopogon* species are the most cited as mosquitoes repellent (Regnault-Roger *et al.*, 2012). Studies on mosquito repellent inventions containing essential oils revealed 144 active patents mostly from Asia especially Chinese, Japanese, Korean and India (Pohlit *et al.*, 2011). Their repellent activity is related to the presence of monoterpenes and sesquiterpenes. However, in some cases, these chemicals can work synergistically, improving their effectiveness (Nerio *et al.*, 2010).

Essential oils from *Cymbopogon nardus* L., *Cymbopogon winterianus* Jowitt ex Bor, *Eucalyptus* species, *Cinnamomum camphora* L., *Cinnamomum zeylanicum* Blume, *Syzygium aromaticum* L., *Pelargonium graveolens*, *Lavandula angustifolia* Mill., *Cymbopogon citratus* DC and peppermint were also described as mosquito repellent or insect repellent (Pohlit *et al.*, 2011). Chemical constituents present in the essential oils with significant mosquito repellent activity include limonene, citronellal, thymol, geraniol, p-menthane-3,8-diol, alpha-pinene, camphor, nepetalactone, citronellol and vanillin. A number of artificial chemicals are still frequently used as repellents, but the essential oils have the potency to provide safer and higher efficient repellent for the environment and mankind (Nerio *et al.*, 2010; Koul & Dhaliwal, 2000). As these essential oils

are highly volatile in nature, their protection time may be reduced. But their efficiency can be significantly increased by mixing with suitable fixatives. That means they can be safely advocated as commercial repellent products (Nerio *et al.*, 2010). Essential oils from *Rosa damascene*, *Pogostemon patchouli*, *Santalum album*, *Lavandula officinalis*, *Pelargonium graveolens* are well-known in perfumery and fragrance industry (Koul *et al.*, 2008).

12.4 Safety of Essential Oils Used in Agriculture

The products derived from plants especially essential oils are an exquisite alternative to non-degradable toxic pesticides as a means to nullify the harmful impacts to the whole mankind and environment. Several essential oils from plants show extensive activity against insects and pathogenic microorganisms as well as protect stored food products. Majority of essential oils are comparatively non-toxic to mammals and fish but it can act as good pesticides. As the essential oils derived from aromatic plants contain many volatile, low-molecular-weight terpenes and phenolics, they are widely used in the field of agriculture as low risk insecticides. Researchers have found out some chemical constituents present in these oils impede with the nervous system of arthropods but not in mammals. The compounds exert some neurotoxic effects on insects through several mechanisms, notably through GABA, octopamine synapses and the inhibition of acetylcholinesterase. While considering all these it is noted that their mammalian toxicity is considerably low and the environmental persistence is short (Regnault-Roger *et al.*, 2012).

In developed countries, several essential oils are used in a series of formulations aimed at handling many arthropods, including spiders, flies, mosquitoes, wasps, gnats, centipedes and moths (Regnault-Roger *et al.*, 2012). The main constituents of these formulations are the five essential oils named peppermint, sesame, thyme, cinnamon and rosemary which got apparently mixed in various proportions. The essential oils extracted from the plant families like *Asteraceae*, *Lamiaceae*, *Myrtaceae* and *Lauraceae*, showed activity against pest insects growth and reproduction (Dev & Koul, 1997; Koul, 1995).

The main chemical constituents of insecticidal essential oils are the monoterpenes. Different chemical ingredients present in essential oils such as, eugenol (*Eugenia caryophyllus*), 1,8-cineole (*Eucalyptus globulus*), citronellal (*Cymbopogon nardus*), pulegone (*Mentha pulegium*), thymol and carvacrol (*Thymus vulgaris*) are the cause of convulsions in insects after being ingested or topically administered. Moreover, EOs can also act as larvicide, antifeedants as well as they are active against insects, mites and nematodes by an octopaminergic mode of action (Dev & Koul, 1997). Essential oils from *Acorus calamus* have been known not only for their insecticidal action but also for the toxic and deterrent effect to variegated cutworms due to the presence of major constituent β -asarone. This compound is oxidised or bioactivated to epoxide in the presence of insect cytochrome p450 enzymes (Koul, 1995).

Essential oils isolated from *Cymbopogon winteriana*, *Eugenia caryophyllus*, *Eucalyptus globulus*, *Thymus vulgaris*, *Rosemarinus officinalis* and *Vetiveria zizanioides* are known for their varied pest control properties (Koul *et al.*, 2008). Similarly, essential oils from *Cymbopogon citratus*, *Acorus calamus*, *Lavandula angustifolia*, *Lavandula officinalis*, *Cinnamomum zeylanicum*, *Tanacetum vulgare*, *Rabdosia melissoides* and *Eugenia*

caryophyllata also exhibited toxicity against different pests (Koul *et al.*, 2008). Moreover, essential oil-bearing plants like *Mentha piperita*, *Melaleuca leucadendron*, *Juniperus virginiana*, *Pelargonium roseum*, *Artemesia vulgaris* and *Lavandula angustifoli* are also effective against various insects and fungal pathogens (Kordali *et al.*, 2005; Koul *et al.*, 2008). These essential oils based pesticides and insecticides may eventually have their ultimate aim in future integrated pest and insect management programme as they are safe to the environment.

12.5 Topical Administration of Essential Oils — Safety Issues

12.5.1 Essential Oils and Aromatherapy

There is some proof regarding the use of essential oils in aromatherapy including various phases of pre- and postoperative treatment. Aromatherapy is successfully established in some economically backward countries due to low cost of this therapy. References about the essential oil aromatherapy give contrasting opinion and Lee *et al.* suggest that 'due to a number of caveats, the evidence is not sufficiently convincing that aromatherapy is an effective therapy for any condition' (Lee *et al.*, 2012). Aromatherapy is the absorption of volatile particles in essential oils through the olfactory mucosa and stimulates olfactory portion of limbic system. These stimuli interact with neuropsychological system to produce physiological and psychological effects. Various applications of aromatherapy using the essential oils are wellbeing, labor, infections, dementia and anxiety treatment while they have not been scientifically validated. Some of the literatures are available for effectiveness of aromatherapy using essential oils such as lavender, peppermint and orange in surgical patients to reduce anxiety and insomnia and to control pain and nausea.

More than 70 patients' cases were reported about the adverse effect of aromatherapy. They described the adverse effect that ranged from minor to serious, including dermatitis and mortality. Essential oils from *Lavandula spica*, *Mentha* species, *Melaleuca* species and *Cananga odourata* showed some adverse effects (Posadzki *et al.*, 2012; Stea *et al.*, 2014). Essential oil from rosemary induced hyperexcitability in cellular levels due to loss of tissue sodium or potassium gradient (Bozorg & Benbadis, 2009). Studies revealed that when a wound was treated with essential oil extracted from tea tree, it showed a reduction in wound healing time. Lavender essential oil was sniffed and applied on the skin of 150 adult patients before surgery and visually analysed the anxiety of patients when they got admitted in pre-surgical suite and also when they got transferred to the operating room. It has been proved that lavender essential oil significantly lower anxiety during operating room transfer (Braden *et al.*, 2009).

Ligusticum chuanxiong essential oil revealed some sort of cutaneous irritation in rabbits. But up to 1 g/kg body weight essential oil had no significant effect on guinea pig skin. Studies showed that the short term application of *Ligusticum chuanxiong* essential oil when used within in the doses is probably safe, but the dose should be under control for external use due to its slight irritation effects (Zhang *et al.*, 2012). Essential oil from *Nepeta cataria* is well known for its mosquito repellent activity against several *Aedes* and *Culex* species. The acute dermal toxicity revealed that LD50 of essential oil was >5000 mg/kg body weight and inhalation LD50 was observed to be >10,000 mg/m³. Moreover, essential oil from *Nepeta cataria* showed moderate skin irritation in New Zealand white rabbits but practically non-irritating to the eye. While considering U.S.

Environmental Protection Agency-approved mosquito repellents like picaridin and p-menthane-3,8-diol, the essential oil from *Nepeta cataria* can be considered as relatively safe, and may cause slight skin irritation (Zhu *et al.*, 2009).

Litsea cubeba oil is an aromatic essential oil showing a cutaneous LD50 and an olfactory LC50 of, approximately, 5000 mg/kg of body weight and 12,500 ppm, respectively (Luo *et al.*, 2005). Cutaneous irritation study in human patients was analysed by using essential oil from *Clausena dentata* which indicated that it is not at all harmful to human skin but with fewer side effects (Rajkumar & Jebanesan, 2010).

12.6 Essential Oils and Eye Safety

Eye irritation toxicity of *Nepeta cataria* (potent mosquito repellent) was performed in rabbits and concluded as practically non-irritating to the eye. Conjunctival irritation was reported 1 h after test material instillation, but did not go beyond 24 h (Zhu *et al.*, 2009). Another study revealed that essential oil from tea tree is used for eyelid scrub treatment which is effective in nullifying demodex infestation and improving subjective ocular symptoms (Koo *et al.*, 2012). Patients with ocular itching and demodex infestation were treated with 5% tea tree oil ointment eye lid massage. The study concluded that there is significant reduction of demodex and ocular itching and the majority of the patients got rid of symptoms like itching (Gao *et al.*, 2012). Another study revealed that the variety of corneal pathological conditions along with conjunctival inflammation, as seen in rosacea, can be found in patients with demodex infection in the eyelids, got significantly reduced by the eyelid scrub with tea tree essential oil (Kheirkhah *et al.*, 2007).

Ophthalmic observation was done after administration of turmeric essential oil in rats at 1 g/kg body weight orally. Ophthalmoscopic observations did not reveal any treatment related changes suggestive of corneal ulcer or retinal vascularity. There was no constriction or extra branching of retinal vessels observed in treated groups and hence common lesions like glaucoma and intraocular inflammatory changes were ruled out as well as this results point out the ocular safety of turmeric essential oil after oral administration (Liju *et al.*, 2013).

12.7 Phototoxicity of Essential Oils

Phototoxicity is a photoirritation otherwise known as chemically induced skin irritation due to the presence of light. Some chemicals absorb light, that changes the molecular nature (photoactive) and cause toxicity. Chemical constituents present in the essential oil may absorb sunlight, increasing the focusing effect of the sun light on skin. Furanocoumarin, a class of phytochemical present in some essential oils, is responsible for the phytophotodermatitis or phototoxicity (Averbeck *et al.*, 1990). Essential oil from lemon contains derivatives from furocoumarin which are well known causative factors for phototoxicity. The compounds producing phototoxicity in this essential oil were identified as being oxypeucedanin and bergapten. Other varieties of citrus-essential oils such as lime oil and bitter-orange oil also contain large amounts of oxypeucedanin (Naganuma *et al.*, 1985). As the essential oil extracted from *Tagetes minuta* and *patula* essential oils are phototoxic in nature, they can't be used to prepare sunscreen products (Scs *et al.*, 2015). In some clinical conditions like lentigo maligna or lentigo maligna

melanoma, the phototoxic property shown by *Citrus bergamia* Risso and *Citrus medica* L. can be advocated (Menichini *et al.*, 2010).

The phototoxicity study in cell line showed, essential oil isolated from Orange (*Citrus aurantium dulcis*) is probably phototoxic in the presence of UV. The essential oil isolated from Lemongrass (*Lemongrass cymbopogon*) is slightly phototoxic in nature. The lemongrass and orange essential oils may have in their chemical constituents one or more factors involved in the phototoxicity reactions. This may be an interesting criteria in the future to study the phototoxicity mechanisms of the molecules involved in cytotoxicity, as well as their targets within the cells (Dijoux *et al.*, 2006).

12.8 Acute and Sub-Chronic Oral Toxicity of Essential Oils

One of the noteworthy features of essential oil is that they are, in general, low-risk products. Their mammalian toxicity is relatively low and they are experimentally well-studied because of their medicinal properties. The acute toxicity of essential oil from *Piper vicosanum* leaves was evaluated by administration to female rats at a single dose of 2 g/kg body weight orally. Signs of acute toxicity were not at all observed, indicating that the LD₅₀ of this oil is >2000 mg/kg (Hoff Brait *et al.*, 2015). Thirty days subchronic toxicity study of Eucalyptus essential oil suggested that high dose (2772 mg/kg b.wt.) reduced the level of male rats growth and histopathology showed that essential oil may cause damage to liver and kidney (Hu *et al.*, 2014). Long-term toxicity study of the essential oil from *Syzygium aromaticum* at a dose of 400 mg/kg, resulted in a significant decrease in body weight but there was no relevant changes in relative organ weights and histopathological analysis. Oral LD₅₀ value of essential oil from *Syzygium aromaticum* was approximately 4500 mg/kg (Liu *et al.*, 2015).

Cupressus lusitanica Mill. essential oil toxicity study showed an elevated serum aspartate transaminase and alanine transaminase levels while the WBC count and protein levels decreased in treated animals at higher dose (Teke *et al.*, 2013). Toxicity tests evaluated in brine shrimp larvae revealed that hemp agrimony (*Eupatorium cannabinum* L.) essential oil containing predominant amounts of germacrene D and neryl acetate were notably toxic (LC₅₀ value 16.3–22.0 µg/mL). Moreover toxicity studies of essential oil from *Litsea cubeba* using brine shrimp larvae that its LC₅₀ values ranges from 25.1–30.9 µL/mL (Judzentiene *et al.*, 2015). Oral LD₅₀ value of the common EOs, such as chamomile, lemongrass, *Lavandula spicaclove*, eucalyptus, anise, and marjoram were ranging from 2000 to 5000 mg/kg body weight in rats. But essential oils from *Artemisia dracuncululus*, *Hyssopus officinalis*, *Origanum vulgare*, *Satureja hortensis*, *Melaleuca alternifolia* and *Sassafras albidum* have LD₅₀ values between 1000 and 2000 mg/kg body weight. Essential oils from *Peumus boldus*, cedar and a mixture of *Mentha pulegium* and *Hedeoma pulegiodes* have LD₅₀ values 130, 830 and 400 mg/kg body weight respectively (Regnault-Roger *et al.*, 2012).

Essential oil from *Schinus molle* showed some lower toxicity in Swiss mice (Martins Mdo *et al.*, 2014). Oral acute toxicity in mice showed that *Croton sonderianus* and *Croton argyrophylloides* oils are nontoxic at lower dose and LD₅₀ >6000 mg/kg body weight while *Crotonnepetaefolius*, *Croton zehntneri* and *Lippia sidoides* essential oils are moderately toxic to mice orally (de Lima *et al.*, 2013). In mice, an oral acute toxicity test revealed that essential oil from *Croton argyrophylloides* can be considered as a nontoxic essential oil since it showed a very high LD₅₀ (9.84 ± 0.01 g/kg) (de França-Neto *et al.*, 2012).

The oral and intra-peritoneal LD₅₀ values of *Ligusticum chuanxiong* essential oil in mice were 7.23 g/kg and 2.25 g/kg respectively. The major constituents present in this essential oil are ligustilide (67.5%) and butylidenephthalide (5.06%) (Zhang *et al.*, 2012). Acute toxicity study of (500 to 4000 mg/kg), *Litsea elliptica* essential oil caused adverse behaviours and mortality in a dose-dependent manner (Budin *et al.*, 2012).

The oral LD₅₀ of essential oil from *Nepeta cataria* was found to be 3160 mg/kg body weight and 2710 mg/kg body weight in female and male rats, respectively (Zhu *et al.*, 2009). Dose-dependent adverse effects of *Cymbopogon citratus*, *Ocimum gratissimum* and *Ocimum basilicum* essential oils were observed during 14 days toxicity study in Wistar rats and resulted in significant functional damages in stomach and liver of Wistar rats (>1500 mg/kg body weight). Unlike other essential oils, administration of *Ocimum gratissimum* oil did not show any adverse effects in rat liver. At recommended concentrations, the three essential oils can be used to store maize since they are safe to human being (Fandohan *et al.*, 2008). Lower concentration of essential oil extracted from the fresh fruits of *Litsea cubeba* is safe. The acute toxicity of *Litsea cubeba* oil in rats reported that the LD₅₀ dose of oral, dermal and inhalation (LC₅₀) are 4000 mg/kg, 5000 mg/kg body weight and 12,500 ppm, respectively in Sprague-Dawley rats (Luo *et al.*, 2005).

Subchronic administration of essential oils from *Minthostachys verticillata*, *Hofmeisteria schaffneri*, *Lippia sidoides* and *Curcuma longa* as well as its formulation with curcumin did not alter the weights, morphology and histopathology, with no adverse effects and mortality. The LD₅₀ values of these essential oils may vary from 3 to 7 g/kg body weight in rats or mice (Escobar *et al.*, 2015; Liju *et al.*, 2013; Fontenelle *et al.*, 2007). Essential oils from *Vitex negundo*, *Crocus sativus*, *Teucrium stocksianum* and *Myrtus communis* had comparatively low toxicity in mice and their LD₅₀ value is over 2000 mg/kg (Chattopadhyay *et al.*, 2014; Mahmoudvand *et al.*, 2015; Costa *et al.*, 2011). Subchronic toxicity reports revealed that NOAEL dosage of *Curcuma longa*, *Zingiber officinale* Roscoe and *Psidium cattleianum* Sabine are 500 mg/kg per day (Liju *et al.*, 2013; Jeena *et al.*, 2011). LD₅₀ of essential oils from different plants and their mode of administration are given in Table 12.1.

12.9 Constituents-Based Toxicity Evaluation of Essential Oils

Usually the chemical constituents present in essential oils have no notable risk related with the oral intake. The toxicity of essential oils mainly depends on the presence of toxic components present in them. Toxicity studies have been done on different chemical ingredients (menthol, carvone, limonene, citral, cinnamaldehyde, benzaldehyde, benzyl acetate, 2-ethyl-1-hexanol, methyl anthranilate, geranyl acetate, furfural, eugeneol and isoeugenol) present in essential oils (Smith *et al.*, 2005). Most of these toxicity studies were done by the U.S. National Toxicology Programme (NTP). Even though they are taken at high levels, the majority of the essential oil constituents showed no carcinogenic effects (Smith *et al.*, 2005). Since these essential oils are highly volatile and lipophilic in nature, they can easily be absorbed into the body via respiratory or cutaneous system and causes significant body effects. Some of the essential oils are photosensitisers (phototoxicity), which means that exposure of essential oil to UV light causes toxicity to skin

Table 12.1 LD50 values of essential oils from different plants and their mode of administration.

No.	Plant name	LD50 concentration	Mode of administration	Animals	References
1	<i>Aegle marmelos L.</i>	23.66g/kg b.wt.	Oral	Mice	Yang <i>et al.</i> (2015)
2	<i>Artemisia dracunculus</i>	1.25 g/kg b.wt.	Intraperitoneal	Rats	Maham <i>et al.</i> (2014)
3	<i>Baccharis dracunculifolia DC</i>	>2g/kg b.wt.	Oral	Rats	Massignani <i>et al.</i> (2009)
4	<i>Croton argyrophylloides</i>	9.8g/kg b.wt.	Oral	Mice	De Franca-Neto <i>et al.</i> (2012)
5	<i>Croton cajucara Benth</i>	9.3g/kg b.wt.	Oral	Mice	Hiruma-Lima <i>et al.</i> (1999)
6	<i>Croton cajucara Benth</i>	680 mg/kg b.wt.	Intraperitoneal	Mice	Hiruma-Lima <i>et al.</i> (1999)
7	<i>Croton nepetaefolius</i>	3,840 mg/kg b.wt.	Oral	Mice	de Lima <i>et al.</i> (2013)
8	<i>Croton sonderianus</i>	>6g/kg b.wt.	Oral	Mice	de Lima <i>et al.</i> (2013)
9	<i>Croton zehntneri</i>	3.5g/kg b.wt.	Oral	Mice	de Lima <i>et al.</i> (2013)
10	<i>Cuminum cyminum</i>	>2g/kg b.wt.	Oral	Rats	Allahghadri <i>et al.</i> (2010)
11	<i>Cupressus lusitanica</i>	6.3g/kg b.wt.	Oral	Mice	Teke <i>et al.</i> (2013)
12	<i>Curcuma longa</i>	<5 g/kg b.wt.	Oral	Rats	Liju <i>et al.</i> (2013)
13	<i>Cymbopogon citratus</i>	3.5g/kg b.wt.	Oral	Mice	Costa <i>et al.</i> (2011)
14	<i>Eucalyptus globulus Labill</i>	3.8g/kg b.wt.	Oral	Rats	Hu <i>et al.</i> (2014)
15	<i>Eucalyptus citriodora</i>	2.7g/kg b.wt.	Oral	Mice	Ribeiro <i>et al.</i> (2014)
16	<i>Eucalyptus staigeriana</i>	3.5g/ml	Oral	Mice	Ribeiro <i>et al.</i> (2015)
17	<i>Eucalyptus oil emulsion in water</i>	3.8g/kg b.wt.	Oral	Rats	Hu <i>et al.</i> (2014)
18	<i>Hofmeisteria schaffneri</i>	>5g/kg b.wt.	Oral	Mice	Angeles-López <i>et al.</i> (2010)
19	<i>Hyptis fruticosa</i>	>5g/kg b.wt.	Oral	Mice	Menezes <i>et al.</i> (2007)
20	<i>Ligusticum chuanxiong Hort.</i>	7.23 g/kg b.wt.	Oral	Mice	Zhang <i>et al.</i> (2012)

(Continued)

Table 12.1 (Continued)

No.	Plant name	LD50 concentration	Mode of administration	Animals	References
21	<i>Ligusticum chuanxiong</i>	2.25 g/kg b.wt.	Intraperitoneal	Mice	Zhang <i>et al.</i> (2012)
22	<i>Lippia graveolens</i>	>5 g/kg b.wt.	Oral	Mice	Rivero-Cruz <i>et al.</i> (2011)
23	<i>Lippia sidoides</i> Cham.	3 g/kg b.wt.	Oral	Mice	Fontenelle <i>et al.</i> (2007)
24	<i>Lippia sidoides</i> oils	2.6 g/kg b.wt.	Oral	Mice	de Lima <i>et al.</i> (2013)
25	<i>Litsea cubeba</i>	4 g/kg b.wt.	Oral	Rats	Luo <i>et al.</i> (2005)
26	<i>Litsea elliptica</i> Blume	3.5 g/kg b.wt.	oral	Rats	Zhang <i>et al.</i> (2012)
27	<i>Mentha longifolia</i> L.	470 mg/kg b.wt.	Oral	Rats	Jalilzadeh <i>et al.</i> (2015a,b)
28	<i>Minthostachys verticillata</i>	>7 g/kg b.wt.	Oral	Rats	Escobar <i>et al.</i> (2015)
29	<i>Myrtus communis</i> L.	2.23 mL/kg b.wt.	Intraperitoneal	Mice	Mahmoudvand <i>et al.</i> (2016)
30	<i>Ocimum basilicum</i>	3.25 g/kg b.wt.	Oral	Rats	Fandohan <i>et al.</i> (2008)
31	<i>Ocimum gratissimum</i>	1.75 g/kg b.wt.	Oral	Rats	Fandohan <i>et al.</i> (2008)
32	<i>Piper vicosanum</i>	<2 g/kg b.wt.	Oral	Rats	Hoff Brait <i>et al.</i> (2015)
33	<i>Poliomintha longiflora</i>	>5 g/kg b.wt.	Oral	Mice	Rivero-Cruz <i>et al.</i> (2011)
34	<i>Psidium cattleianum</i>	>500 µg/ml b.wt.	Oral	Mice	Castro <i>et al.</i> (2015)
35	<i>Salvia libanotica</i>	839 mg/kg b.wt.	Intraperitoneal	Mice	Farhat <i>et al.</i> (2001)
36	<i>Syzygium aromaticum</i>	4.5 g/kg b.wt.	Oral	Rats	Liu <i>et al.</i> (2015)
37	<i>Vitex agnus-castus</i>	<5 g/kg b.wt.	Oral	Mice	Khalilzadeh <i>et al.</i> (2015)
38	<i>Vitex negundo</i>	<2 g/kg b.wt.	Dermal toxicity	Rats	Chattopadhyay <i>et al.</i> (2014)
39	<i>Zataria multiflora</i> Boiss.	1264.9 µL/kg b.wt.	Intraperitoneal	Rats	Majlessi <i>et al.</i> (2012)
40	<i>Zingiber officinale</i> Roscoe	<2 g/kg b.wt.	Oral	Rats	Jeena <i>et al.</i> (2011)

(neroli, rosemary, cassia, calamus, and bitter almond). Mostly phototoxicity of essential oils are caused by the presence of psoralens or furanocoumarins (Klarmann, 1958; Lis-Balchin, 2006). Psoralen is the compound present in *Citrus bergamia* essential oil which bind to DNA, producing adduct and causes cytotoxic and mutagenicity. Some of the fragrance constituents present in essential oils may cause allergy related problems (Balsam of Peru) (Ford, 1991). Moreover, it was documented that Wormwood essential oil caused human acute renal failure (Maistro *et al.*, 2010). Furthermore, reports suggested that essential oils and their major components rarely created genotoxicity in animals. Methyl Eugenol, estragole, safrol and asarone are the essential oil constituents that are generally regarded as carcinogens.

Limonene, the major constituent of citrus essential oil is a common industrial cleaning agent. Human exposure of d-Limonene causes allergic contact dermatitis and decrease in the lung vital capacity at highest doses (Falk Filipsson *et al.*, 1993; Lis-Balchin, 2006). Moreover, cats and dogs are highly susceptible to insecticides and baths containing D-limonene giving rise to neurological symptoms such as ataxia, stiffness, apparent severe CNS depression, tremors and coma (Lis-Balchin, 2006). The main chemical constituent of sassafras oil is safrole, which had been used in perfume industry and in food since ancient times. But now it is limited to 1 mg/kg in foods due to toxicity (Council Directive 92/109/EEC, 14 December 1992) as well as it is listed as category 1 substances, since they are precursors for illicit manufacture of hallucinogenic, narcotic and psychotropic drugs (Lis-Balchin, 2006). Moreover thujone in immense quantities can cause lesions in cerebral cortex along with convulsions (Keith & Starraky, 1935) and it is allowed at 0.5 mg/kg in foods (Lis-Balchin, 2006).

12.10 Genotoxicity and Carcinogenicity of the Essential Oils

Essential oils are usually devoid of mutagenicity and carcinogenicity in lower concentration. But some other essential oils or their chemical constituents may act as carcinogen after metabolic activation (Guba, 2001). *Salvia sclarea* and *Melaleuca quinquenervia* essential oils stimulate oestrogen production and may induce oestrogen dependent malignancy. Essential oils from *Ocimum basilicum* and *Artemisia dracunculus* contain estragole, which showed carcinogenic potential in rodent (Miller *et al.*, 1983; Anthony *et al.*, 1987). *Citrus bergamia* oil contain photosensitising compound such as Psoralen which may induce skin cancer after DNA adduct formation in the presence of sunlight or ultraviolet radiations (Averbeck *et al.*, 1990; Averbeck & Averbeck, 1998). Pulegone and safrole are main chemical constituents commonly present in essential oils from mint, *Sassafras albidum* and *Ocotea pretiosa*, may induce cancer after metabolic activation (Zhou *et al.*, 2004; Miller *et al.*, 1983; Burkey *et al.*, 2000; Liu *et al.*, 2000). Methyl Eugenol is a chemical constituent from *Laurus nobilis* and *Melaleuca leucadendron* essential oil and D-limonene, a monoterpene found in citrus essential oil has already been reported to be carcinogenic in rodents (Burkey *et al.*, 2000; NTP, 1990).

Usually mutagenicity and antimutagenicity studies of essential oils are mainly conducted on bacteria (*Salmonella typhimurium*, *Bacillus subtilis* and *Escherichia coli*), mammalian cells, and insects (*Drosophila melanogaster*). Studies on these came to the conclusion that essential oils can damage the mitochondrial DNA which leads to mitochondrial dysfunction and respiratory deficient (Bakkali *et al.*, 2008). Essential oils indirectly or directly defect energy metabolism and respiration. Mitochondrial and

respiratory metabolism changes were observed after treatment of tea tree essential oil with *Saccharomyces cerevisiae* (Abraham *et al.*, 2003).

Studies of essential oil from *Piper vicosanum* showed no genotoxicity and mutagenicity in mice. While conducting the comet assay and micronuclei test, this essential oil did not increase the frequency or rate of DNA damage in mice peripheral blood erythrocytes (Hoff Brait *et al.*, 2015). Micronuclei and comet assay studies of *Minthostachys verticillata* essential oil revealed that up to a concentration of 7 g/kg feed for 90 days did not produce a cyto-genotoxic effect on the bone marrow and peripheral blood cells of Wistar rats (Escobar *et al.*, 2015). Genetic toxicity of *L. cubeba* oil was assessed with *Salmonella typhimurium*, by determination of the induction of micronuclei in bone marrow cells, and also by testing for chromosome aberration in spermatocyte cells and results of genotoxicity testing of *L. cubeba* oil *in vitro* and *in vivo* were negative (Luo *et al.*, 2005). Studies revealed that turmeric essential oil isolated from turmeric had not produced any mutagenicity to *Salmonella typhimurium* with or without metabolic activation. Moreover genetic aberration or micronuclei in rat bone marrow cells and comet assay confirming the non-genotoxicity of turmeric essential oil up to 1 g/kg body weight in rats (Liju *et al.*, 2013). Genotoxicity study of lemongrass essential oil in male Swiss mice showed no genotoxic effect from the *Cymbopogon citratus* EO at 100 mg/kg body weight.

Studies on somatic mutations in *Drosophila melanogaster*, recombination test (SMART), sister chromatid exchange test and the chromosomal aberration test on human lymphocytes suggested that *Mentha spicata*, *Pinus sylvestris*, *Mentha piperita* and *Anethum graveolens* essential oil are genotoxic (Franzios *et al.*, 1997, Karpouhtsis *et al.*, 1998, Lazutka *et al.*, 2001). Mentone isolated from pepper mentha essential oil gave affirmative results in Ames test and genotoxic in SMART test. Likewise, anethol from fennel and anise essential oils and asarone isolated from *Acorus calamus* essential oil showed the same (Andersen & Jensen, 1984; Franzios *et al.*, 1997; Nestman & Lee, 1983; Hasheminejad & Caldwell, 1994; Goggelmann & Schimmer, 1983). Genotoxicity in Ames test was also shown by terpineol, trans-anethole oxide and trans-asarone oxide. Moreover these constituents also induce hepatic and dermal malignancy (Kim *et al.*, 1999; Gomes-Carneiro *et al.*, 1998). The compounds such as cinnamaldehyde, carvacrol, thymol and carvone, isolated from different essential oils, also exhibited alteration of the nucleotide sequence of the genome in Ames test (Stammati *et al.*, 1999). Eugenol showed genotoxic and chromosomal aberration in cells (Maralhas *et al.*, 2006). High dose of *Anethum graveolens*, *Pinus densiflora* and peppermint essential oil have been reported that they were cytotoxic and genotoxic for human lymphocytes (Lazutka *et al.*, 2001).

12.11 Conclusion

Because of a series of potential multipurpose benefits, essential oils bagged world wide acceptance. Although essential oils are widely used in the field of agriculture and industry, they leave behind lower risk to environment and human health than synthetic chemicals. Even though the essential oils are safe, toxicological studies revealed that some of them may be harmful to human health, if not used carefully, as they are highly concentrated and have multiple constituents. Moreover, it is important that whatever be the therapeutic benefits we expect from the essential oils, it is necessary to evaluate

the safety efficacy of them before legally marketed for food or drugs. For this, *in vivo* experimentation must be carried out. Besides these, the toxicity profile evaluations of essential oils are highly useful to explore the therapeutic, scientific, economic and conventional uses of essential oils.

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